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Identification des gènes impliqués dans la production et la détoxication des espèces activées de l'oxygène chez *Hevea brasiliensis* et leur caractérisation dans le latex

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Identification des gènes impliqués dans la production et la détoxication des espèces activées de l'oxygène chez *Hevea brasiliensis* et leur caractérisation dans le latex

Résumé

Hevea brasiliensis, un arbre tropical, est la source principale de caoutchouc naturel commercialement viable. La biosynthèse de caoutchouc se passe dans les cellules spécialisées appelées laticifères. Il représente jusqu'à 90% de la matière sèche du latex. Le latex, laiteux, s'écoule de l'encoche faite sur l'écorce de l'arbre jusqu'aux cellules laticifères. La collecte de latex, faite par saignée régulière tous 2-3 jours, est vraiment stressante pour l'arbre. Pour stimuler la production de latex, un générateur d'éthylène peut être appliqué sur le panneau de saignée. Le stress s'intensifie avec l'application hormonale. La production d'espèces activées de l'oxygène (ROS) se fait en réponse aux stress environnementaux ainsi que lors de l'exploitation de l'arbre. Au-delà d'un certain seuil, la production de ROS est massive dans les laticifères. De nombreuses études ont montré que les ROS entraînent une dégradation par peroxydation des lipides insaturés des membranes et ensuite une déstabilisation et lyse des organites. La lyse des lutoïdes permet la libération des facteurs de coagulation dans le latex entraînant la coagulation *in situ* des particules de caoutchouc dans l'écorce des arbres stressés. Ce syndrome physiologique, appelé syndrome de l'encoche sèche (TPD), est un des facteurs limitant la production de caoutchouc.

Ce travail de thèse vise à identifier les gènes associés à la production et la neutralisation des ROS ainsi que leurs caractérisations dans les laticifères. Premièrement, nous avons fait une analyse bibliographique complète sur les gènes associés à la production et la neutralisation des ROS chez l'hévéa et les plantes modèles. La NADPH oxydase a été décrite comme la source principale de ROS chez les arbres stressés. Les enzymes antioxydantes et les antioxydants constituent le système de neutralisation des ROS. Deuxièmement, à partir d'une analyse à l'échelle du génome, 407 gènes impliqués dans la production des ROS et dans leur neutralisation ont été identifiés. Troisièmement, à partir d'une analyse du transcriptome, 164 gènes redox ont été détectés dans le latex du clone SP 217 et 161 dans celui du clone PB 260. Quatrièmement, à partir des petits ARNs et d'une analyse dégradome, 13 gènes ont été identifiés pour être clivés par 11 microARNs et 15 gènes clivés par 16 petits ARNs phasés dans le latex. Enfin, cette étude a mis en évidence des régulations spécifiques de la production des ROS et du système antioxydant dans le latex. *HbRBOH2* a été identifié comme la source principale de ROS dans le latex. *HbCuZnSOD4* pourrait être le contributeur majeur de la neutralisation des ROS dans le latex des arbres atteints de TPD.

Mot-clefs: *Hevea brasiliensis*, syndrome de l'encoche sèche, latex, espèces activées de l'oxygène, respiratory burst oxidase homologue, polyphenol oxidase, superoxide dismutase, peroxidase, catalase, methionine sulfoxide reductase, peroxiredoxine, glutaredoxine, thioredoxine, glutathion, ascorbate, tocotriénol

Identification of genes involved in the production and scavenging of reactive oxygen species in *Hevea brasiliensis* and their characterizations in latex

Abstract:

Hevea brasiliensis, a tropical tree, is the main commercial source of nature rubber. The rubber biosynthesis occurs in specialized latex cells of rubber tree. Up 90% dry weight of latex is nature rubber. The milky latex flows out from cut latex cells by tapping rubber tree trunk bark. Rubber exploitation by tapping every several days is very stressful for the bark of rubber tree. To stimulate latex production, ethylene releaser is applied during rubber exploitation in some cases. The stress is increased after hormone stimulation. Reactive oxygen species (ROS) is generated when plant suffers stresses from environment and harvesting activity. Over a certain limit of stress, ROS bursting is motivated in latex cell. A lots of the evidences showed that the ROS lead to the peroxidatic degradation of the unsaturated lipids of the membrane and then to destabilisation and lysis of the organelles. Lysis of the lutoids results in liberation of coagulating factors into latex and coagulation *in situ* of rubber particles in stressed trees. This serious physiology syndrome is tapping panel dryness (TPD) which is one of main factor limiting rubber production.

This PhD aims at identifying ROS production and scavenging genes and their characterizations in latex cell. Firstly, we made a comprehensive bibliography study on ROS production and scavenging genes both in rubber tree and model plant. The NADPH oxidase was considered as the main source of ROS in the stressed trees. ROS scavenging enzymes and antioxidants constituted the ROS scavenging systems in latex. Secondly, based on a genome-wide analysis, 407 genes involved in ROS production and scavenging were identified. Thirdly, based on a transcriptome analysis, 164 redox-related genes were detected expressing in latex of clone SP217 and 161 genes expressing in latex of clone PB260. Fourthly, based on small RNA and degradome analysis, 13 genes were shown to be targeted by 11 microRNAs and 15 genes by 16 phased siRNA in latex. Lastly, this study illustrated specific regulation systems of ROS production and scavenging in latex. *HbRBOH2* was identified as the main source gene of ROS in latex. *HbCuZnSOD4* might be the most important ROS scavenging gene to detoxify the ROS in latex of TPD tolerant tree.

Key words: *Hevea brasiliensis*, tapping panel dryness, latex, reactive oxygen species, respiratory burst oxidase homolog, polyphenol oxidase, superoxide dismutase, peroxidase, catalase, methionine sulfoxide reductase, peroxiredoxin, glutaredoxin, thioredoxin, glutathione, ascorbate, tocotrienol

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Résumé substantiel

Identification des gènes impliqués dans la production et la détoxication des espèces activées de l'oxygène chez *Hevea brasiliensis* et leur caractérisation dans le latex

1. *Hevea brasiliensis*

Hevea brasiliensis, l'arbre à caoutchouc, est un arbre tropical appartenant à la famille des *Euphorbiaceae*. C'est la source principale et économiquement biable de caoutchouc naturel (NR). Originaire du bassin amazonien, l'hévéa est principalement cultivé en Asie du Sud-Est pour la production de caoutchouc naturel depuis plus d'un siècle. Après 5 ou 6 ans, l'arbre à caoutchouc est ouvert pour récolter le latex pendant 30 à 40 ans en saignant l'écorce du tronc tous les 2-3 jours. Le latex laiteux est le cytoplasme contenant du caoutchouc et plus de 90% du poids sec de latex est le NR. Le NR (cis-1,4-polyisoprène) a été largement utilisé pour des produits industriels pour son élasticité, son excellente isolation, son imperméabilité et ses caractéristiques de plasticité. La production et la consommation de caoutchouc ont augmenté au cours des dernières décennies, à l'exception d'une légère diminution de la période de crise financière mondiale de 2008 à 2009. Les plantations d'hévéa se composent principalement de greffons clonaux greffés sur des porte-greffes. Les principales maladies des arbres à caoutchouc sont la maladie sud-américaine des feuilles, l'oïdium, la maladie de feuilles liée à *Corynespora* et au *Colletotrichum*, 'bird's eye spot', maladies de racine et le syndrome de l'encoche sèche.

Le saccharose est la source de carbone et d'énergie de la biosynthèse du caoutchouc dans le latex, cytoplasme des laticifères. La biosynthèse de l'isopentényl diphosphate (IPP) est le précurseur de la biosynthèse du caoutchouc. Le saccharose est transformé en pyruvate par glycolyse dans le cytosol. Il existe deux voies de synthèse de l'isopentényl diphosphate (IPP) dans le latex. Ce sont la voie du 2-C-méthyl-D-érythritol-4-phosphate (MEP) dans les plastides et du mévalonate (MVA) dans le cytosol. Six enzymes sont impliquées dans la voie MEP, qui convertit le pyruvate en IPP. Six enzymes sont impliquées dans la voie MVA, qui convertit l'acétyl-CoA en IPP. Le pyruvate est transformé en citrate dans les mitochondries par deux enzymes: la pyruvate déshydrogénase et la citrate synthase. Le citrate cytosolique est converti en acétyl-CoA par l'ATP-citrate lyase. L'IPP peut être convertie en plusieurs unités de *cis*-isoprène via 4 enzymes: IPP isomérase (IPPI), géranyl diphosphate synthase (GPS), géranyl géranyl diphosphate synthase (GGPS) et farnésyl diphosphate synthase (FPS). Des unités de *cis*-isoprène multiples (y compris IPP) sont ajoutées dans des molécules de *cis*-1,4-polyisoprène via la cis-prényltransférase (CPT). Le facteur d'élongation du caoutchouc (REF) et la petite protéine de particules de caoutchouc (SRPP) sont nécessaires pour réguler l'allongement des chaines.

La production de latex dépend de son flux et de sa régénération entre deux saignées. Le flux de latex est contrôlé par la pression de turgescence et l'afflux d'eau à l'intérieur des laticifères à partir des tissus du phloème après la saignée. Il est récolté en blessant les tissus mous de l'écorce avant le lever du soleil en raison de la transpiration moindre et de la pression plus élevée des laticifères tôt le matin, ce qui permet au latex de s'écouler plus facilement et

dans un plus grand volume. La régénération du latex dépend principalement de la disponibilité et du métabolisme du saccharose et de la limitation des activités enzymatiques impliquées dans la régénération du latex et des composés azotés. Pour les clones de l'hévéa à faible métabolisme, l'éthéphon, un agent de libération de l'éthylène, est appliqué sur le panneau de saignée pour stimuler la production de latex. L'application d'éthéphon induit plusieurs changements biochimiques chez les laticifères, tels que la charge en saccharose, l'absorption d'eau, l'assimilation d'azote ou la synthèse de protéines de défense, impliquant un grand nombre de gènes de réponse à l'éthylène.

2. Les espèces actives de l'oxygène dans le latex d'*Hevea brasiliensis* et leurs implications dans le syndrome de l'encoche sèche

L'encoche sèche (TPD), un syndrome physiologique, est l'un des facteurs limitant le plus important de la production de latex. La sensibilité à la TPD dépend de la génétique, de l'interaction entre le porte-greffe et le greffon et des stress abiotiques / biotiques. Il résulte normalement de la surexploitation des hévéas, y compris une fréquence de saignée élevée et / ou une trop forte stimulation avec l'éthéphon, responsable d'une perte importante de production de caoutchouc. La production d'espèces activées de l'oxygène (ROS) et le piégeage ont lieu dans des laticifères en réponse au stress de récolte et en réponse à l'activité métabolique conséquente nécessaire à la régénération du latex après saignée. Au-delà d'un certain seuil, une quantité massive ROS intra-laticifère est générée. Un grand nombre de preuves ont montré que les ROS conduisent à la peroxydation des lipides insaturés des membranes puis à la déstabilisation et à la lyse des organites. La lyse des lutoïdes entraîne la libération des facteurs coagulants dans le latex entraînant la coagulation *in situ* des particules de caoutchouc dans les arbres stressés. Ce syndrome physiologique grave est le syndrome de l'encoche sèche (TPD) qui est l'un des principaux facteurs limitant la production de caoutchouc.

La NADPH oxydase, localisée à la surface des lutoïdes d'hévéa, est considérée comme la source principale de ROS et produit des ions superoxydes ($O2^{\bullet -}$) *via* l'oxydation du NADPH dans le latex. La sous-unité enzymatique clé de la NADPH oxydase végétale est codée par le gène *respiratory burst oxidase homolog* (*Rboh*), qui est considéré comme le «moteur» de la signalisation des ROS dans la plante. Il y a deux fortes corrélations entre l'activité de NADPH oxydase des lutoïdes et l'éclatement des lutoïdes ; et entre l'éclatement des lutoïdes et la production de caoutchouc. Les lutoïdes provenant d'arbres sains, à rendement élevé et à rendement moyen ne présentaient que des traces, lorsqu'elle était détectable, de l'activité de la NADPH oxydase. Les lutoïdes provenant d'arbres à très faible rendement, avec aucun symptôme visible d'encoche sèche, a montré une activité NADPH oxydase faible mais détectable. Seuls les arbres présentant des symptômes visibles d'encoche sèche présentaient une forte activité NADPH oxydase.

La NADPH oxydase pourrait être grandement activée par des concentrations physiologiques de cations métalliques et de composés apparentés à la quinone dans les laticifères. La plupart des cations métalliques tels que Cu^{2+}, Ca^{2+} et Mg^{2+}, plastoquinone et ubiquinol sont normalement compartimentés dans les lutoïdes. Ils pourraient agir comme

activateurs ou transporteurs d'électrons pour la NADPH oxydase. Une force de cisaillement sur la membrane des lutoïdes est causée par l'écoulement du latex et une diminution de la pression osmotique causée par le réapprovisionnement en eau des cellules environnantes des laticifères. Ces deux facteurs physiques inévitables auraient pour conséquence une lyse des lutoïdes. Certains cations métalliques libérés activeraient la NADPH oxydase et augmenteraient la production de ROS dans les laticifères. L'oxydation des lipides, de l'ARN, de l'ADN et des protéines du latex, provoquée par ROS, se produirait si les ROS n'étaient pas correctement neutralisées par la CuZnSOD, la catalase, les peroxydases et la peroxiredoxine dans le cytoplasme. D'autre part, le phénol cytosolique pourrait être oxydé en quinone par le peroxyde d'hydrogène qui activerait de nouveau la NADPH oxydase. Si les enzymes neutralisant les ROS ne peuvent pas correctement neutraliser ces ROS dans l'arbre stressé, l'oxydation de la membrane des lutoïdes s'étend et s'aggrave. Il y a alors une augmentation de l'éclatement de lutoïdes accompagnant le burst oxydatif dans les laticifères. Des facteurs coagulants abondamment libérés dans le cytoplasme aboutissent au TPD chez les arbres stressés. Si la production massive de ROS continue après la coagulation au caoutchouc, la linamarine présente dans l'écorce est libérée. La linamarase transforme la linamarine en cyanure, ce qui entraîne une nécrose de l'écorce de l'arbre stressé.

Le système antioxydant joue un rôle crucial dans le contrôle du flux de latex et de la production de latex qui en découle chez *Hevea brasiliensis*. L'activité de la NADPH oxydase est très faible lorsque le système neutralisant les ROS est totalement efficace dans le latex provenant d'arbres sains non stressés. Les systèmes antioxydant dans le latex protègent les laticifères contre les ROS, et sont constitués d'enzymes et de molécules antioxydantes. Un modèle de travail sur les systèmes antioxydants dans le latex d'hévéa a été explicité. Le tocotriénol, les caroténoïdes, les phénols, le glutathion et l'ascorbate sont les principaux antioxydants du latex. L'activité cuivre-zinc superoxyde dismutase (CuZnSOD) constitue la première ligne de défense contre les ROS en convertissant l'$O_2^{\bullet -}$ en H_2O_2 dans le cytosol. Le peroxyde d'hydrogène (H_2O_2) est converti en H_2O par l'ascorbate peroxydase (APx), la glutathion peroxydase (GPx), la peroxydase (Px), la peroxiredoxine (Prx) et la catalase (Cat) dans le cytosol. Puisque la catalase a une affinité beaucoup plus faible pour l'H_2O_2, l'ascorbate peroxydase peut piéger l'H_2O_2 plus efficacement que la catalase à de faibles concentrations d'H_2O_2. En revanche, la catalase est une enzyme très puissante à des concentrations élevées d'H_2O_2 car une molécule de catalase peut décomposer des millions de molécules de peroxyde d'hydrogène en oxygène et en eau. Les étapes du cycle Ascorbate-Glutathion reposent sur les électrons fournis par les composés réducteurs de faible poids moléculaire, tels que l'ascorbate et le glutathion. Dans le cycle Ascorbate-Glutathion, H_2O_2 est réduit en H_2O par APx et GPx en utilisant respectivement de l'ascorbate et du glutathion comme donneur d'électrons. La monodéshydroascorbate réductase (MDHAR) et la déhydroascorbate réductase (DHAR) transfèrent l'énergie du NADPH et du GSH à l'ASA, respectivement. La glutathion réductase (GR) réduit le disulfure de glutathion (GSSG) en utilisant le NADPH comme donneur d'électrons. L'énergie réductrice de Prx vient du NADPH. A partir de certaines réactions intermédiaires, le NADPH est consommé pour réduire le peroxyde d'hydrogène. La thiorédoxine réductase (NRT) dépendante du NADPH et la thiorédoxine (TRX) forment la voie la plus importante pour transmettre l'énergie du NADPH au Prx ou au GPX. Les électrophiles

sont détoxifiés par la glutathion S-transférase (GST). L'énergie nécessaire à la neutralisation des ROS et à la détoxification des électrophiles (lipides oxydés ROS, ADN et phénol) est générée par l'oxydation du NADPH. La réaction globale de la production de ROS et de la neutralisation des ROS dans les laticifères est l'oxydation du NADPH avec de l'oxygène. Grâce à la consommation de NADPH, le stress a été perçu et transduit par un système d'homéostasie complexe de production et de neutralisation des ROS dans les laticifères.

Plusieurs analyses transcriptomiques ont montré une régulation transcriptionnelle des gènes redox, mais nous sommes loin d'une compréhension globale de la régulation mise en jeu. L'analyse fonctionnelle des systèmes redox nécessitera une intégration des informations protéomiques et métabolomiques.

3. L'analyse des gènes redox à l'échelle du génome chez *Hevea brasiliensis* laticifers révèle des régulations post-transcriptionnelles fortes et spécifiques de l'espèce.

Avec une étude bibliographique complète des systèmes redox dans le latex d'hévéa, nous avons trouvé beaucoup de données physiologiques et biochimiques étudiées et quelques gènes liés au redox clonés dans le latex dans le passé. Les expressions de certains gènes liés au redox ont été rapportées par RNAseq dans le latex. Toutes ces études ont globalement analysé l'expression génique, mais n'ont pas vérifié spécifiquement toutes les familles de gènes liés au redox. L'étude sur les systèmes redox d'hévéa était finalement peu fouillée.

Maintenant, la disponibilité de la séquence du génome de l'hévéa et l'analyse de l'expression génique à haut débit ouvrent la perspective d'identifier tous les gènes impliqués dans la production de ROS, la régulation, la neutralisation et la biosynthèse des antioxydants chez l'hévéa et leurs expressions dans le latex. Nous avons d'abord réalisé une étude bibliographique sur les gènes liés à la gestion du statut redox chez Arabidopsis (gènes impliqués dans la production de ROS, la régulation, le piégeage et la biosynthèse des antioxydants) et téléchargé leurs séquences protéiques à partir de la base de données UniProt. Ensuite, nous avons fait un tblastn contre le génome et le transcriptome de l'hévéa à l'aide de bioinformaticiens et sur Galaxy. Les résultats de tblastn ont généré des milliers de séquences liées aux réactions d'oxydo-réduction. Après l'identification complète des cadres ouverts de lecture (ORF) par blastx sur NCBI sur chacune de ces séquences, l'analyse phylogénétique des ORFs de ces gènes validés a été réalisée par Mega 6 avec leurs séquences protéiques. Enfin, les gènes liés au redox chez l'hévéa ont été identifiés et leurs expressions dans le latex ont été étudiées. Pour étudier de manière exhaustive les systèmes redox dans le latex, la localisation subcellulaire les protéines redox a été prédite à l'aide de trois outils. De plus, des microARN ciblant des gènes liés au redox s'exprimant dans le latex ont été validés par Shuangyang Wu et Julie Leclercq *via* le pipeline Cleaveland avec la base de données dégradome et une base de données de petits ARNs. Les transcrits redox, clivés ou non par un microARN, et s'exprimant dans le latex ont été ensuite analysés par RNAseq chez un clone d'hévéa sensible au TPD dans diverses conditions de récolte de latex et affecté ou non par le TPD. Cette étude a fourni un aperçu de la coordination

transcriptionnelle et post-transcritionnelle du système oxydant-antioxydant en relation avec la tolérance au stress de récolte de latex et au TPD.

Le système de piegage des ROS dans le latex est complexe. La détermination des principaux gènes neutralisant les ROS parmi 161 gènes s'exprimant dans le latex a été le point clé pour établir une stratégie de modification génitique et pour le développement de marqueurs génétiques moléculaires. Il y avait une base de données de transcriptome généré par l'équipe à partir du séquençage 454 d'ARN provenant de 6 tissus de l'hévéa (écorce, embryon, latex, feuille, tissus reproducteurs et racine) chez le clone PB260. Malheureusement, ce séquençage n'était pas assez profond pour effectuer une analyse statistique, mais était sufisant pour vérifier l'expression des gènes liés aux réactions d'oxydo-réduction dans les différents tissus. Tous ces gènes liés au statut redox s'exprimant dans le latex n'étaient spécifiques du latex. Nous avons trouvé quelques lectures dans les autres tissus. Cependant, il y avait 22 gènes exprimés en abondance dans le latex comparés à d'autres tissus: *HbPPO1, HbGPx6, HbMSRA2, HbMSRB2, HbPRX2B2, HbMGST, HbGrxC2, 13 HbTrxs, HbVTE2* et *HbVTC22*. Les peroxydases sont exprimées très faiblement dans le latex avec seulement quelques lectures détectées. En revanche, ils sont exprimés à un niveau très élevé dans d'autres tissus: 1162 et 559 lectures d'HbPx1 ont été détectées respectivement dans l'écorce et la racine ainsi que 788, 1991 et 536 lectures d'HbPx5 dans l'écorce, l'embryon et la racine.

Comme pour le clone PB260 étudié et sensible au TPD, il y avait des données RNAseq provenant du clone tolérant SP217. La limite de ces données était que l'échantillon de l'arbre sain après le traitement par l'éthéphon manquait en raison de la faible qualité de l'ARN. Le latex du clone SP217 a été prélevé tous les jours alors que celui de PB260 l'était tous les 2 jours. En raison de la tolérance au TPD, les arbres TPD du SP217 ont été induits uniquement par une fréquence de saignée plus élevée. Par conséquent, SP217 et PB260 n'ont pas été échantillonnés dans le même état de stress. Le SP217 a été échantillonné sous un stress plus important. Pour éviter que la faiblesse de ces données déroute les lecteurs, nous n'avons pas mis les données d'expression génique du clone SP217 et dans les différents tissus de PB260 dans l'article publié dans le chapitre consacré. Cependant, l'étude de l'expression des gènes liés au redox dans latex du clone SP 217 est une bonne référence pour la conception de l'expérimentation à venir. Deux comparaisons entre les deux clones sont possibles: arbre sain après traitement de l'eau (W / H) et arbre TPD après traitement par l'éthéphon (ET / TPD). Les profils d'expression des gènes liés au redox dans ces deux clones étaient très différents. En effet, 11 et 12 gènes liés au statut redox sont exprimés respectivement à des niveaux plus élevés et plus faibles, à la fois chez les arbres sains et atteints de TPD du clone SP 217. En outre, 17 et 21 gènes liés au statut redox sont exprimés à des niveaux plus élevés et plus faibles respectivement chez les arbres sains du clone SP217. Enfin, 8 et 6 gènes liés au statut redox sont exprimés respectivement à des niveaux plus élevés et moins élevés chez les arbres atteints de TPD du clone SP 217. Il y a 3 autres gènes de peroxydase exprimés dans le latex du clone SP 217: *HbPx19, HbPx22* et *HbPx53*. *HbPx22* et *HbPx53* ont été localisées dans l'espace extracellulaire et *HbPx19* a une localisation inconnue.

Les systèmes de production et de piégeage des ROS jouent un rôle essentiel dans le maintien de l'homéostasie redox dans les laticifères. Outre les études sur les espèces modèles

végétales, cette étude est l'analyse la plus complète à l'échelle du génome des systèmes de production et de piégeage des ROS et de la biosynthèse des antioxydants dans une culture pérenne. Sur la base d'une analyse à l'échelle du génome, 407 gènes impliqués dans la production et le piégeage de ROS ont été identifiés chez l'hévéa. C'est un nombre plus élevé de gènes comparé à ceux d'Arabidopsis. Un nouveau modèle de travail a été proposé pour les régulations transcriptionnelles et post-transcriptionnelles en tenant compte de la localisation subcellulaire prédite des protéines déduites. Sur la base d'une analyse du transcriptome, 164 gènes liés au redox ont été détectés et s'expriment dans le latex du clone SP 217 et 161 gènes exprimant dans celui du clone PB 260. Sur la base d'une analyse dégradome et des petits ARNs, 13 gènes sont ciblés par 12 microARNs et 15 gènes par 16 siARN phasés dans le latex.

Cette étude a illustré des systèmes de régulation spécifiques de la production et du piégeage des ROS dans le latex. NTR-TRX-Prx pourrait être la principale voie de piégeage du H_2O_2 et la GSH-GST pourrait être la principale voie de détoxification des produits oxydés dans le latex, uniquement basé sur la quantité des membres d'une famille de gènes et sur l'abondance transcrits trouvés dans le latex. Les ARNm de 13 *HbTRX* étaient très abondants dans le latex parmi les 6 tissus étudiés et *HbTRXH5* était le transcrit le plus abondant dans le clone PB 260. Outre *HbRBOH2* et *CuZnSOD4*, les gènes impliqués dans les voies des NTR-TRX-Prx, GSH-GST et dans la biosynthèse des antioxydants n'avaient pas un changement d'expression aussi important entre deux clones ou n'étaient pas des gènes s'exprimant abondamment dans le latex. *HbRBOH2* était le principal gène de production de ROS exprimés dans le latex du clone PB 260, ainsi que dans celui du clone SP 217. Il était plus faiblement exprimé dans le latex de SP217 par rapport à celui PB260. Il a été identifié comme le gène codant la source principale de ROS chez les arbres stressés. *CuZnSOD4* est le principal gène SOD exprimé dans le latex de SP217 mais pas dans PB260 car il est exprimé à un niveau plus élevé dans SP 217 que dans PB 260. *HbCuZnSOD4* pourrait être le gène piégeur de ROS le plus important pour détoxifier le ROS dans le latex d'un arbre tolérant au TPD. Les différences clonales ou de fréquence de saignée pourraient provoquer les changements du niveau d'expression importants (0,44 / 6,56, 223,97 / 26,40 et 130,09 / 5,27) de *HbRBOH2* et de *HbCuZnSOD4* observés entre les deux clones. Une nouvelle expérience est nécessaire pour valider le facteur qui conduit à une modification de l'expression des gènes. À l'inverse d'Arabidopsis, aucune des isoformes *CuZnSOD cytosoliques* d'hévéa n'est soumise à une régulation post-transcriptionnelle par miR398.

4. Etablissement d'un système, à l'échelle du génome, de découverte des gènes cibles d'un facteur de transcription pour HbERFs basé sur la transformation stable

Le générateur d'éthylène (Ethephon) est largement appliqué pour stimuler la production de latex. C'est la seule hormone végétale qui peut induire aussi bien la production de latex que le TPD, ce qui indique que la voie de signalisation de l'éthylène peut être impliquée dans la régulation du système redox dans le latex d'hévéa pour déclencher le TPD. RRTF1 est le nœud principal du réseau de co-expression et qui contrôle un régulon en réponse à un changement du statut redox. *HbERF-Xb1* est le seul orthologue de AtRRTF1 chez l'hévéa, est fortement

exprimé dans le latex après la saignée et la stimulation par l'éthéphon. D'autres ERFs pourraient jouer un rôle dans le réseau de régulation du statut redox de l'hévéa, famille qui a été la plus étudiée étant donné le rôle de l'éthylène dans la production de caoutchouc. Les candidats ERF impliqués dans la régulation des systèmes redox présentés sont: *HbERF-Ib5, HbERF-IXc4, HbERF-IXc5, HbERF-Xb1, HbERF-VIIa12* et *HbERF-VIIa17*.

Pour l'étude des gènes cibles des ERFs, TARGET est la technologie la plus appropriée pour l'étude d'interaction entre les ERFs d'hévéa et leurs gènes cibles. TARGET, dans sa version originale, est un système de transformation transitoire pour la découverte de cibles de facteur de transcription à l'échelle du génome, dans lequel la transformation du protoplaste végétal est essentielle. Ce système de découverte de cibles de facteur de transcription à l'échelle du génome sera adapté aux lignées de cals transgéniques de HbERFs d'hévéa, qui peuvent être obtenues de façon routinière par notre équipe. Cette adaptation du système TARGET à l'hévéa et l'application de cette approche pour étudier les cibles des HbERF a été réalisée. L'analyse du transcriptome après l'induction du promoteur permettra l'identification des gènes cibles primaires et secondaires en présence ou en l'absence de cycloheximide. Le domaine GR permet l'entrée contrôlée de la protéine chimère GR-ERF dans le noyau par l'addition du ligand du domaine GR, la dexaméthasone (DEX). Le gène de la bêta-glucuronidase (GUS) a été choisi comme gène de contrôle et a confirmé la fiabilité du système d'expression.

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Abbreviation

3, 4 D	3, 4-dichlorophenoxy-acetic acid
ABA	Abscisic acid
acetyl-CoA	Acetyl coenzyme A
APx	Ascorbate peroxidase
ASC	Ascorbate
At	*Arabidopsis thaliana*
BI	Lutoids brusting index
B-serum	Bottom serum
Ca	Cambium
Cat	Catalase
cDNA	Complementary DNA
CHIP-seq	Chromatin immunoprecipitation sequencing
CHX	Cycloheximide
Cp	Conducting phloem
CPT	*cis*-prenyltransferase
C-serum	Clear serum
CuZnSOD	Cu/Zn superoxide dismutase
Cys	Cysteine
DEG	Differential expressed gene
DEX	Dexamethasone
DHA	Dehydroascorbate
DHAR	Dehydroascorbate reductase
DM	Decontamination medium
DMSO	Dimethylsulphoxide

DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ERF	Ethylene response factor
ET	Ethylene
FPS	Farnesyl diphosphate synthase
Gb	Giga base pairs
GCL	Glutamate cysteine ligase
GDH	L-galactose dehydrogenase
GFP	Green florescence protein
GGPS	Geranyl geranyl diphosphate synthase
GLDH	L-galactono-1,4-lactone dehydrogenase
Gly	Glycine
GME	GDP-mannose 3,5-epimerase
GPS	Geranyl diphosphate synthase
GPx	Glutathione peroxidase
GR	Glutathione reductase
GR	Glucocorticoid receptor
Grx	Glutaredoxin
GS	Glutathione synthetase
GSH	Glutathione
GSSG	Glutathione disulfide
GST	Glutathione S-transferase
GULO	L-gulonolactone oxidase
GUS	beta-glucuronidase
H	Healthy
H_2O_2	Hydrogen peroxide

HAC	Acetic acid
Hb	*Hevea brasiliensis*
HLL	Lutoid membrane lectin-like protein
IPP	Isopentenyl diphosphate
IPPI	Isopentenyl diphosphate isomerase
JA	Jasmonate
Kb	Kilo base pairs
La	Laticifer
LD	Liquid medium
MDHA	Monodehydroascorbate
MDHAR	Monodehydroascorbate reductase
MEP	2-C-methyl-D-erythritol-4-phosphate
Miox	Myo-inositol oxygenase
miRNA	microRNA
MM	Maintenance culture
MnSOD	Mn superoxide dismutase
mRNA	Messenger RNA
MSR	Methionine sulfoxide reductase
MVA	Mevalonate
NADPH	Nicotinamide adenine dinucleotide phosphate
NR	Natural rubber
Nrx	Nucleoredoxin
NTR	NADPH dependent thioredoxin reductase
$O^{\bullet}$	Singlet oxygen
$O2^{\bullet-}$	Superoxide radicals
$O^{\bullet}3$	Ozone

$^{\bullet}OH$	Radical hydroxyl
OD	Optical density
pH	Potential Hydrogen
PPO	Polyphenol oxidase
Prx	Peroxiredoxin
Px	Peroxidase
Ra	Rays
RBOH	Respiratory burst oxidase homolog
REF	Rubber elongation factor
RNA	Ribonucleic acid
RNAseq	RNA sequencing
ROS	Reactive oxygen species
rpm	Rounds per minute
RRTF1	Redox regulation transcription factor 1
SNP	Single nucleotide polymorphism
SR	Synthetic rubber
SRPP	Small rubber particle protein
SSR	Simple sequence repeat
T-DNA	Transfer-DNA
TF	Transcription factor
TPD	Tapping panel dryness
Trx	Thioredoxin
UV-B	Ultraviolet B
VTC1	GDP-D-mannose pyrophosphorylase
VTC2	GDP-L-galactose phosphorylase
VTC4	Inositol phosphate phosphatase

VTE1	Tocopherol cyclase
VTE2	Homogentisate phytyltransferase
VTE3	MPBQ/MSBQ methyltransferase
VTE4	Tocopherol gamma-methyltransferase
W	Water
X-Gluc	5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid
Xy	Xylem
γ-EC	γ-glutamylcysteine

General Introduction

5. *Hevea brasiliensis*

5.1 Biology

Hevea brasiliensis, the Para rubber tree, is a tropical tree belonging to the *Euphorbiaceae* family. It is a tall vigorous tree growing to a height of up to 30 to 40 meters (Figure 1) with conic taproot and verticillate lateral root. The trunk is cylindrical and may have a swollen, bottle-shaped base (Figure 1). The leaves have three elliptic leaflets and are spirally arranged. Rubber tree is monoecious and its flowers are unisexual in the panicles. Both male and female flowers are found in the inflorescence and female are located at the top of the branch and are larger than male flowers. Fruit capsules normally contain three seeds inside of the woody and hard endocarp. The seed is oval and brown, and when the capsule is mature, the nut bursts out automatically and the seed pops up. The surface of bark is brown and the inner bark oozes latex when damaged. (Information from Plants of the World online: http://powo.science.kew.org/taxon/urn:lsid:ipni.org:names:349913-1#image-gallery.)

Figure 1. Immature rubber tree plantation.

5.2 Rubber tree plantation

Coming from the Amazonian basin, rubber tree is mainly cultivated in Southeast Asia (Figure 5) for natural rubber (NR) production more than one century (Mooibroek and Cornish 2000). Over the last several decades more than one million hectares of land have been converted to rubber trees in areas of China, Laos, Thailand, Vietnam, Cambodia and Myanmar (Li and

Fox 2012). Rubber plantations consist mostly of clonal scion plants grafted on rootstock produced from seedling material. Because of its reproduction biology, rootstocks are different genetically. After 5 or 6 years old (Figure 1), the rubber tree is open to harvest the latex by tapping the bark of trunk in the early morning one time per several days. Because of the milky latex, NR is above 90% dry weight of latex (Jacob et al. 1993), rubber tree is the most economically and primary source of NR.

5.3 Laticifer

Laticifers, in the inner bark of rubber tree, is the latex producing cells (Figure 2). They are connected by anastomosis (Hao 1982). Two types of laticifers, primary and the secondary laticifers in *Hevea* bark, which are based on the corresponding stages of laticifer growths (Hao and Wu 2000a). The secondary laticifers are the principal type of laticifers exploited commercially for its latex. Its number is relevant to rubber production and is determined by laticifer differentiation, which is controlled genetically and also influenced by environmental condition (Hao and Wu 2000b; Zhang et al. 2015b).

Besides of nucleus, ribosome, mitochondrion, cytoplasm, plastid, vacuome and walls, rubber particle, lutoid and Frey-Wyssling particle were identified as three kinds of specialized organelles in laticifer (Southorn 1960; Dickenson 1969). Rubber particles, natural rubber synthesis and storage organelles, form 30% to 45% of fresh latex volume and 90% of latex dry weight (Jacob et al. 1993). Their size varies from 50Å to 3 μm in diameter (Gomez and Hamzah 1989). They are surrounded by a half-unit biomembrane (Wood and Cornish 2000) and a large number of proteins (Dai et al. 2013b). Lutoid is a vacuole which is enclosed by a unit biomembrane. It is a lysosomal vacuome in latex (d'Auzac et al. 1982a). Lutoids form 1 to 5 μm in diameter and nearly 20% of fresh latex volume (d'Auzac et al. 1982a). Numerous proteins and chemical elements are enclosed in lutoids, some of them are involved in the regulation of rubber particle aggregation and latex coagulation (Wang et al. 2013). Frey-Wyssling particles, which are carotenoid containing chromoplasts and enveloped by a double biomembrane, form 4 to 6 μm in diameter and 2% to 3% of fresh latex volume (Dickenson 1969; Jacob et al. 1993). They contained polyphenol oxidase (Tata and Edwin 1970). Polyphenol oxidase was involved in the dandelion latex coagulation (Wahler et al. 2009). Therefore Frey-Wyssling particle might be involved in the *Hevea* latex coagulation.

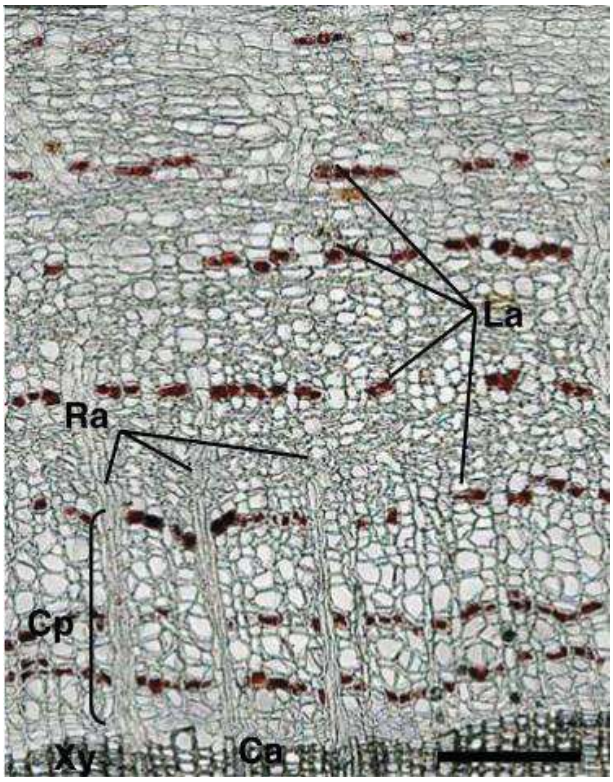

Figure 2. *Hevea brasiliensis* laticifer in the bark (Sando et al. 2009). Ca, cambium; La, laticifers; Ra, rays; Xy, xylem; Cp, conducting phloem. Scale bar is 200 μm.

6. Worldwide rubber production and consumption

Rubber (cis-1, 4-polyisoprene) has been used widely to process industrial products in daily life for its elasticity, excellent insulation, impermeability and plasticity characteristics. After proper treatment, rubber has oil resistance, acid and alkali resistance, heat resistance, cold resistance, pressure resistance, wear resistance and other valuable properties. It is processed for tires, gloves, condoms, bags, elastic bands, belts, hoses, balloons, etc. Besides the NR from rubber tree, synthetic rubber (SR) is produced artificially and more easily from polymers to mimic the properties of NR. NR and SR are not fully substitutable, especially in tire manufacturing which is the primary rubber consumption.

Although the demand for NR has continued increasing, the world production of SR was exceeding NR since 1960s (Mooibroek and Cornish 2000) (Figure 3). The rubber production and consumption have been increasing during the last decades in general (Figure 3-4). There was only a slightly decreasing period from 2008 to 2009, which was during the world financial crisis (Figure 3-4).

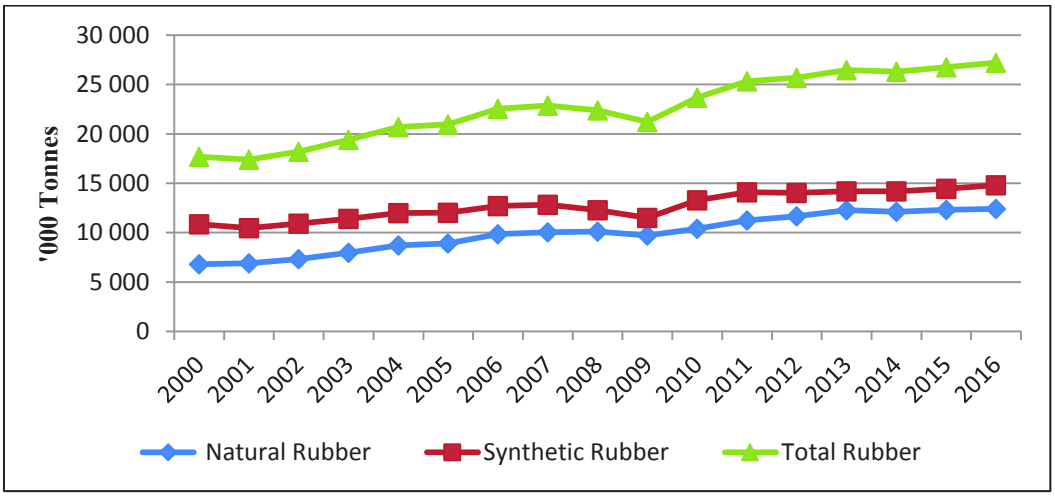

Figure 3. World rubber production from 2000 to 2016. (Data from International Rubber Study Group).

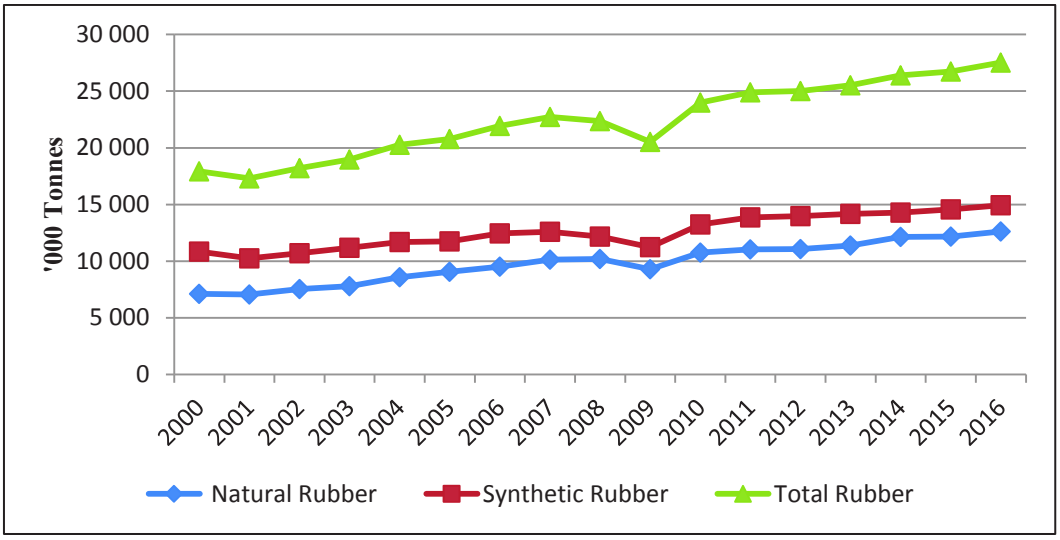

Figure 4. World rubber consumption from 2000 to 2016. (Data from International Rubber Study Group).

Rubber tree is cultivated mainly in Southeast Asia but consumed by the whole world (Figure 5). As the world's largest consumer and fifth producer (Figure 5), China consumed 4.8 million tons of natural rubber in 2016, accounting for 38% of the total NR production (Yongkang 2017). Because of the serious imbalance between supply and demand, China mostly imports natural rubber to meet the additional demand.

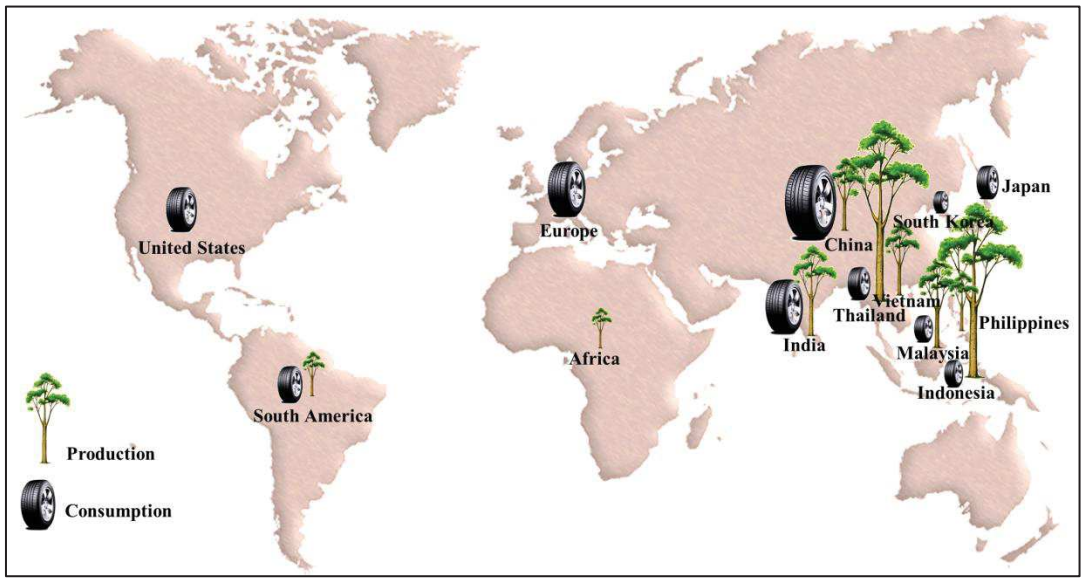

Figure 5. Natural rubber production and consumption main regions or countries. (Data from Food and Agriculture Organization of the United Nations database which is updated until 2014)

7. Rubber biosynthesis and latex harvesting

7.1 Rubber biosynthesis

The rubber biosynthetic pathway occurs in laticifer (Figure 6). Sucrose is the carbon and energy source of rubber biosynthesis. Isopentenyl diphosphate (IPP) biosynthesis is the precursor of rubber biosynthesis (Cornish and Xie 2012). Sucrose is converted into pyruvate *via* glycolysis in cytosol. There are two pathways for isopentenyl diphosphate (IPP) synthesis in latex (Tang et al. 2016). They are 2-C-methyl-D-erythritol-4-phosphate (MEP) pathway in plastid and mevalonate (MVA) pathway in cytosol. Six enzymes, encoded by 12 genes, are involved in MEP pathway, which converts pyruvate into IPP. Six enzymes, encoded by 18 genes, are involved in MVA pathway, which converts acetyl-CoA into IPP. Pyruvate is converted into citrate by two enzymes, pyruvate dehydrogenase and citrate synthase, in mitochondria. Cytosolic citrate is converted into acetyl-CoA by ATP-citrate lyase. IPP can be converted into multiple cis-isoprene units *via* 4 enzymes. They are IPP isomerase (IPPI), geranyl diphosphate synthase (GPS), geranyl geranyl diphosphate synthase (GGPS) and farnesyl diphosphate synthase (FPS) (Cornish et al. 1993; Cornish and Xie 2012; Tang et al. 2016). Multiple cis-isoprene units (including IPP) are added into cis-1, 4-polyisoprene molecules *via cis*-prenyltransferase (CPT). CPT is encoded by 11 genes in *Hevea* latex (Tang et al. 2016). Both rubber elongation factor (REF) and small rubber particle protein (SRPP) are necessary to regulate the elongating reaction (Dennis and Light 1989; Oh et al. 1999). REF and SRPP share a conserved REF motif and they are encoded by 18 genes in *Hevea* latex (Tang et al. 2016).

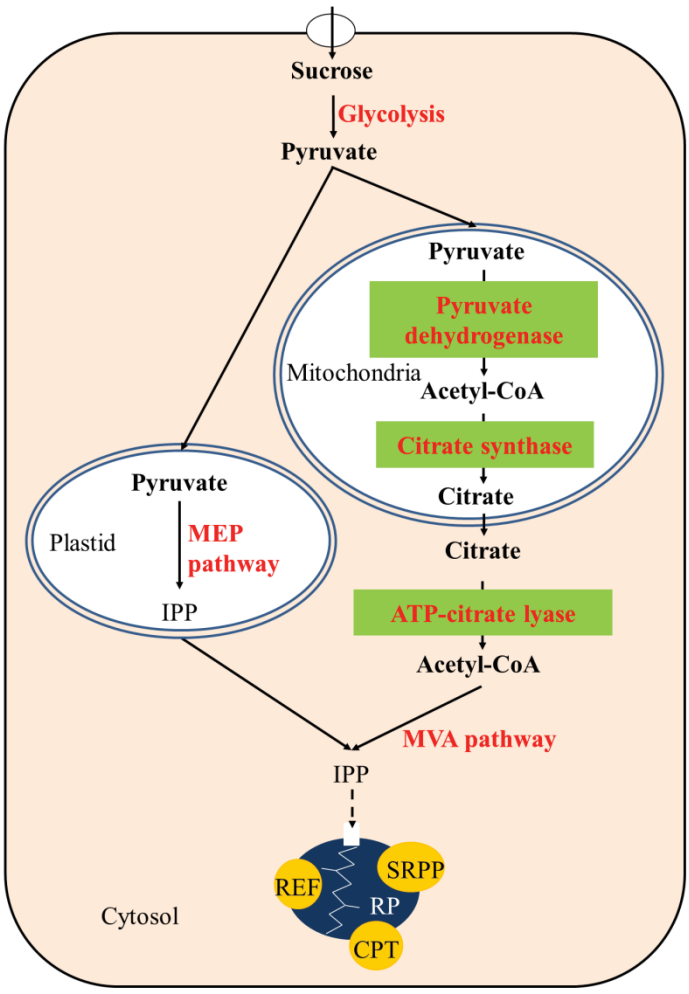

Figure 6. Biosynthesis of natural rubber in *Hevea brasiliensis* (Tang et al. 2016). RP, rubber particle.

7.2 Latex harvesting

Latex, a rubber-containing cytoplasm, is harvested by tapping the soft bark tissues (Figure 7.b-c). The latex is harvested every several (2-5) days in the early morning because of less transpiration and higher pressure in latex cell make the latex flow out easier and in a larger volume.

For *Hevea* clones with a low latex metabolism, ethephon, an ethylene releaser, is applied to the tapping panel to stimulate latex production (Figure 7.a). Ethephon application induces several biochemical changes in laticifers, such as sucrose loading (Dusotoit-Coucaud et al. 2009; Dusotoit-Coucaud et al. 2010), water uptake (Tungngoen et al. 2009), nitrogen assimilation or synthesis of defence proteins (Hadrami and D'auzac 1992), involving a large number of ethylene-response genes (Piyatrakul et al. 2012; Kuswanhadi et al. 2010; Duan et al. 2010; Pujade-Renaud et al. 1994; Tang et al. 2010; Zhu and Zhang 2009). Production of endogenous ethylene by tapping and exogenous ethylene by ethephon stimulation are likely to be sources of stress conducive to the biosynthesis of defence proteins and secondary metabolites comprising rubber in order to protect wounded laticifers (Kush et al. 1990b).

(a) (b) (c)

Figure 7. Latex harvesting. (a) Ethephon stimulation; (b) Tapping; (c) Latex flow along the tapping cut to a cup.

8. Limiting factors of natural rubber production

Rubber plantations are affected by biotic and abiotic stress, including fungus infection, cultivated clones varieties, soil fertility, typhoon, rainfall, atmospheric temperature, etc (Rao et al. 1990; Raj et al. 2005). Main rubber tree diseases are South American leaf blight, powdery mildew disease, *Corynespora* leaf disease, *Colletotrichum* leaf disease, bird's eye spot, root diseases (http://rubberdisease.blogspot.fr/2010/08/rubber-diseases.html) and tapping panel dryness. The annual growth cycle of *Hevea* is affected by climatic factors and soil factors. Abiotic stress depends on locational factors of plantation. For instance, in South of China, rubber trees are planted only in tropical or close to tropical area. Cold acclimation and typhoon cause damages on the rubber tree and lastly affect latex production.

Latex production depends on its flow and regeneration between two tappings (Jacob JL 1989). Latex flow is controlled by turgor pressure and water influx inside the laticifers from phloem tissues after tapping (Tungngoen et al. 2011). Latex regeneration mainly depends on the availability and metabolism of sucrose and limiting enzymatic activities involved in latex regeneration (Dusotoit-Coucaud et al. 2009) and of nitrogen compounds (Pujade-Renaud et al. 1994; Hadrami and D'auzac 1992). Additionally, the dynamic balance between reactive oxygen species generation and scavenging regulated both latex flow and regeneration.

9. Tapping Panel Dryness

Tapping Panel Dryness (TPD), a physiological syndrome, is one of the most important limiting factor of latex production. It normally results from overexploitation of rubber trees including high tapping frequency or/and ethephon stimulation, is responsible of important loss of rubber production (Moraes and Moraes 2010; Okoma et al. 2011a; de Faÿ 2011). TPD susceptibility depends on genetic susceptibility, rootstock-scion interaction and abiotic/biotic stress. Two kinds of typical TPD syndromes are observed which depends on the degree of TPD, due to *in situ* coagulation of rubber particles (Putranto et al. 2015b). They are partial cessation of latex flow related to reactive oxygen species-Tapping Panel Dryness (Figure 8.b) and total cessation of latex flow related to brown bast-TPD (Figure 8.c) (Putranto et al. 2015b).

(a) (b) (c)

Figure 8. Various level of latex flowing from healthy and Tapping Panel Dryness-affected trees. (a) Healthy tree; (b) Dry cut tree; (c) Brown bast tree.

10. Reactive oxygen species (ROS) production and scavenging related to Tapping Panel Dryness (TPD)

10.1 Plant ROS

In plant, the main ROS includes ozone ($O^{\bullet}_3$), singlet oxygen ($O^{\bullet}$), superoxide radicals ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and radical hydroxyl ($^{\bullet}OH$) (Foyer and Noctor 2009). Most of them are very reactive and short lived in cells and generates oxidative damages to lipids, proteins and nucleic acids. Besides their functions involving in plant development and signal transduction (Karkonen and Kuchitsu 2015; Baxter et al. 2014b; Foyer and Noctor 2005a), ROS production plays a crucial role in abiotic/biotic stress response in plant (Pospisil 2016; Zhang et al. 2016a; Huang et al. 2016).

10.2 ROS production in *Hevea* latex

ROS production and scavenging take place in laticifers in response to harvesting stress and consequent metabolic activity necessary for latex regeneration after tapping (Chrestin et al. 1984). NADPH oxidase, localized on the membrane of *Hevea* lutoid, which is considered as the main source of ROS and produces superoxide radicals (O2•-) *via* oxidation of NADPH in latex. The key enzymatic subunit of plant NADPH oxidases is encoded by the Respiratory burst oxidase homolog (Rboh) gene (Suzuki et al. 2011), which is considered as the “engine” of ROS

signalling. There were two strong correlations between lutoid NADPH oxidase activity and the bursting of lutoids and between the bursting of lutoids and the production of rubber (Chrestin 1989a). After centrifugation of Tapping Panel Dryness latex, there is no or fewer lutoids observed comparing to healthy one. Lutoids from healthy, high and medium yielding trees exhibited only traces, when detectable, of NADPH oxidase activity. Lutoids from very low-yielding trees no obvious symptoms of typical Tapping Panel Dryness showed low but detectable NADPH oxidase activity. Only trees with obvious symptoms of Tapping Panel Dryness exhibited fully effective NADPH oxidase activity.

10.3 Dry cut is a ROS-TPD

The ROS-TPD is due to *in situ* latex coagulation. Latex coagulation is controlled and regulated by a variety of proteins (Figure 9). Over a certain limit of environmental and harvesting stresses, an intra-laticifer oxidative burst is generated. ROS lead to the peroxidatic degradation of the unsaturated lipids of the membrane and then to destabilisation and lysis of the organelles. Lysis of the lutoids, and to subsequent liberation into the latex of the coagulating factors which results in latex coagulation *in situ* of the stressed trees. Released hevamine contacts membrane-localized glucanase which facilitates rubber particle aggregation (Wang et al. 2013). Hevein, released from lutoid, is involved in the *in situ* coagulation of rubber particles (Gidrol et al. 1994a). But this type of aggregation is not directly related to the association observed between lutoid membrane debris and rubber particles (Wititsuwannakul et al. 2008b). A rubber particle protein and a lutoid membrane lectin-like protein are involved in rubber particles aggregation and latex coagulation (Wititsuwannakul et al. 2008c; Wititsuwannakul et al. 2008b). This hem-aggregation is inhibited by C-serum lectin binding protein that is important physiologically for maintaining the colloidal stability by preventing the coagulation of the latex (Wititsuwannakul et al. 2008a). A cytosolic Hev b 7-like protein also inhibited rubber particles aggregation during ethrel treatment (Shi et al. 2016). Released chitinase from lutoids may play a role in removing the sugar moiety from small rubber particle protein which inhibits the rubber particle aggregation (Wang et al. 2013).

10.4 Brown bast is a variant of TPD

Brown bast, or bark nercrosis, is a variant of TPD. This degenerative disease of rubber tree trunk phloem was considered induced by local release of a high concentration of easily diffusive cyanide after decompartmentalization near the root stock/scion junction (Chrestin et al. 2004; Nandris et al. 2005) (Figure 9). Because cyanide is known to be produced in the plant cell and to affect cell respiration (Solomos and Laties 1976). Linamarase could be released from the lutoids, a specific vacuole, after lutoids bursting in *Hevea* laticifer (Wang et al. 2013) (Figure 9). Although latex itself exhibited very low (if any) cyanide potential, it is quite high in trunk inner bark of mature trees (Kongsawadworakul et al. 2009). The largest amount of hydrogen cyanide released by trunk bark tissues decreases latex pressure potential resulting in brown bast (Moraes et al. 2014; Faÿ et al. 2010).

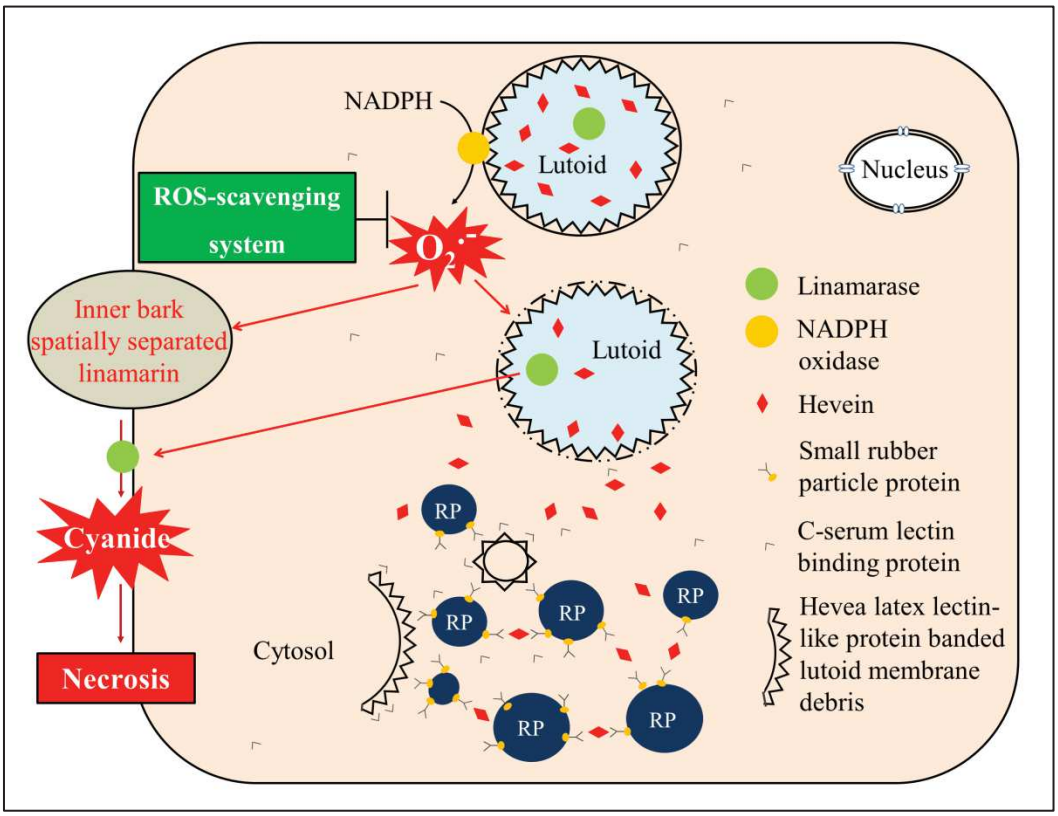

Figure 9. Scheme of molecular mechanisms involved in rubber coagulation and TPD in latex based on several publications (Gidrol et al. 1994a; Kongsawadworakul et al. 2009; Wititsuwannakul et al. 2008a; Wititsuwannakul et al. 2008b; Wititsuwannakul et al. 2008c; Wang et al. 2013; Moraes et al. 2014; Faÿ et al. 2010).

10.5 ROS scavenging systems in latex

Activation of the ROS-scavenging system, including ROS-scavenging enzymes and antioxidants, is very important in abiotic/biotic stress response of rubber tree. Latex thiol is an antioxidant, its contents determining latex yield and cold tolerance (Alam et al. 2003). Peroxidase activity has been suggested to play a major role in the mechanisms of rubber trees defend against biotic stress (Geiger et al. 1989). Peroxidase activity increases in rubber tree tissues to overcome ROS accumulation upon fungus infection (Geiger et al. 1976; Geiger et al. 1989). Activation of the ROS-scavenging system, especially genes encoding peroxidase and superoxide dismutase, was reported under high light intensity and a water deficit (Putranto et al. 2012; LiFeng 2014a, b). Rubber plants grown with a water deficit accumulated proline and ascorbate, and although most enzymes with antioxidant activities were stable, glutathione reductase activity dramatically increased in line with the water deficit (Sanier et al. 2013).

The ROS-scavenging system plays a crucial role in the control of latex flow and consequent latex production in *Hevea brasiliensis*. NADPH oxidase activity is very low when the ROS-scavenging system is fully efficient in the latex from healthy non-stressed trees. A working model of ROS-scavenging systems in *Hevea* latex was drawn by our group based knowledge on plant (Maurino and Flugge 2008) (Figure 10). NADPH oxidase generates superoxide radicals ($O_2^{\bullet-}$) *via* oxidation of NADPH. Copper-zinc superoxide dismutase (CuZnSOD) activity constitutes the first line of defence against ROS by converting $O_2^{\bullet-}$ into

H_2O_2 in cytosol. Hydrogen peroxide (H_2O_2) is converted into H_2O by ascorbate peroxidase (APx), glutathione peroxidase (GPx) and catalase (Cat) in cytosol. Since catalase has a far lower affinity for H_2O_2 (Halliwell 1974), ascorbate peroxidase can scavenge H_2O_2 more efficiently that catalase at low H_2O_2 concentrations. By contrast, catalase is a very potent enzyme at high H_2O_2 concentrations because one molecule of catalase can decompose millions of hydrogen peroxide molecules into oxygen and water. Ascorbate-glutathione cycle steps rely on electrons supplied by reducing compounds of low molecular weight, such as ascorbate and glutathione (Noctor and Foyer 1998b). In the glutathione-ascorbate cycle H_2O_2 is reduced to H_2O by APx and GPx using ascorbate and glutathione as the electron donor respectively. Monodehydroascorbate reductase (MDHAR) and dehydroascorbate reductase (DHAR) transfer reducing energy from HADPH and GSH to ASA respectively. Glutathione reductase (GR) reduces glutathione disulphide (GSSG) using NADPH as the electron donor. GSH biosynthesis is carried out by two critical enzymes: glutamate cysteine ligase (GCL) and glutathione synthetase (GS). γ-glutamylcysteine (γ-EC) is synthesized from L-glutamate (Glu) and cysteine (Cys) *via* a ligation reaction by GCL. Then, glycine (Gly) is added to the C-terminal of γ-EC *via* GS.

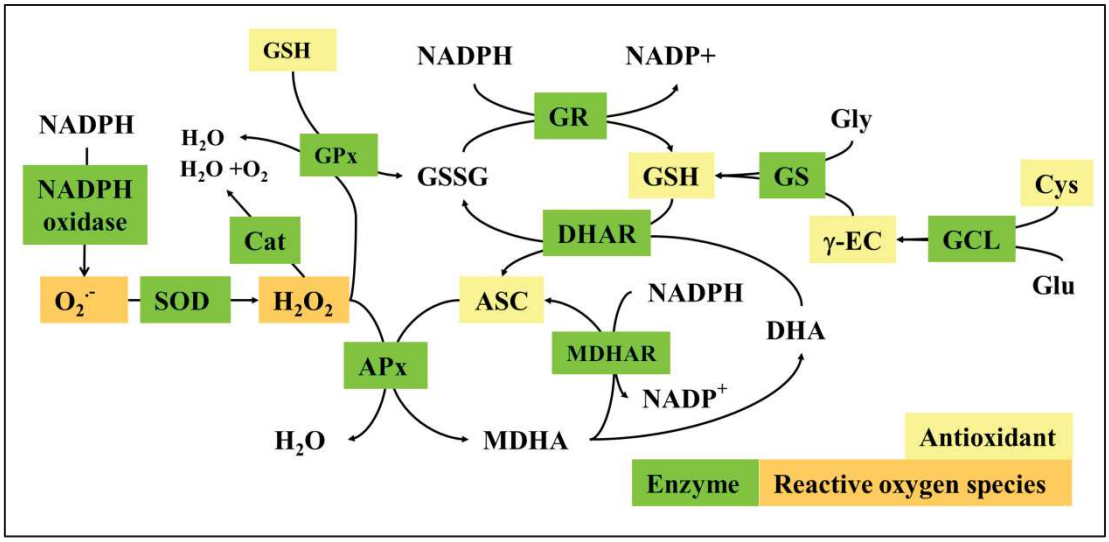

Figure 10. Working model of ROS-scavenging systems in *Hevea* latex based knowledge on plant (Maurino and Flugge 2008).

10.6 Ascorbate biosynthesis in plant

There are 3 ascorbate biosynthesis pathways evidenced in plant (Ishikawa et al. 2006) (Figure 11). One pathway is control by Myo-inositol oxygenase (Miox) and L-gulonolactone oxidase (GULO). Miox converts Myo-Inositol into D-Glucuronate in cytosol. After several reactions, D-Glucuronate is converted into L-Gulono-1, 4-lactone (L-GulL). GULO converts L-Gulono-1, 4-lactone into ASA in plastid. There are 6 rate-limiting enzymes in another ascorbate biosynthesis pathway. GDP-D-mannose pyrophosphorylase (VTC1) and GDP-L-galactose phosphorylase (VTC2) converts D-Mannose-1-P (D-M-P) into GDP-D-Mannose (GDP-D-M). GDP-mannose 3, 5-epimerase 1 (GME) converts GDP-D-M into GDP-L-Galactose (GDP-L-Gal). VTC2 converts GDP-L-Gal into L-Galactose-1-P (L-Gal-P). Inositol

phosphate phosphatase (VTC4) converts L-Gal-P into L-Galactose (L-Gal). L-galactose dehydrogenase (GDH) converts L-Gal into L-Galactono-1, 4-lactone (L-GalL). These 5 reactions occur in cytosol. L-galactono-1, 4-lactone dehydrogenase (GLDH) converts L-GalL into ASA in plastid.

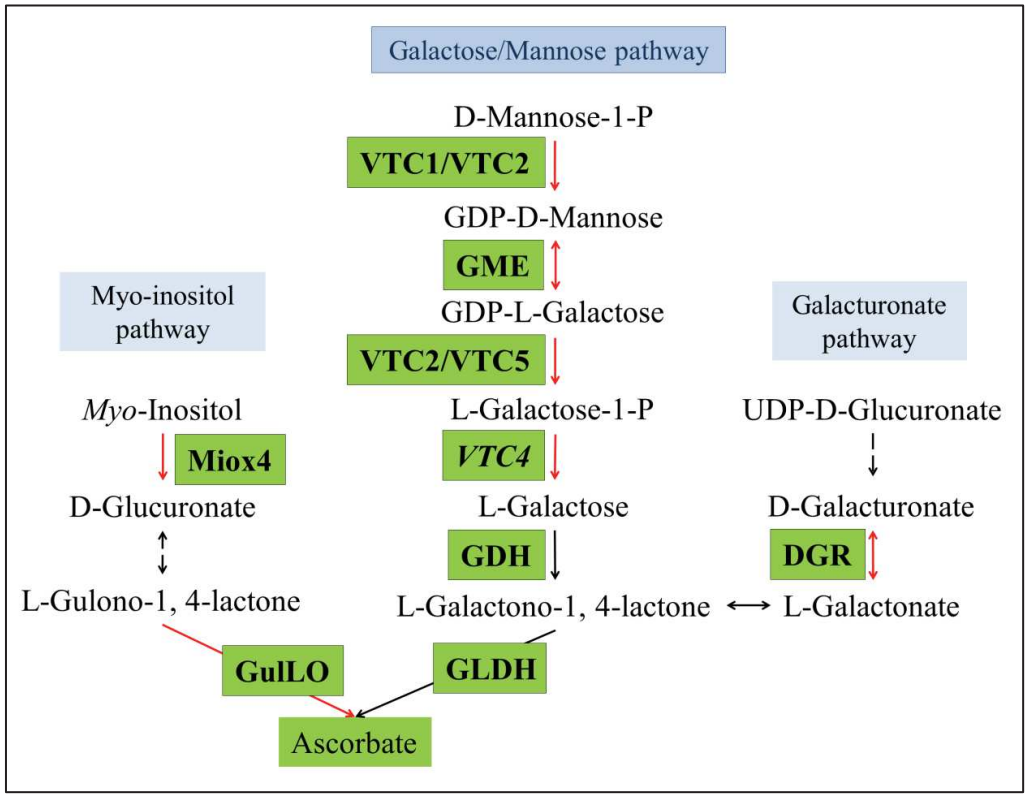

Figure 11. Ascorbate (ASA) biosynthesis in plant. Scheme modified from (Ishikawa et al. 2006).

10.7 Tocotrienol biosynthesis in plant

Besides GSH and ASA, tocotrienol is another important antioxidant in latex. As lipid-soluble antioxidants, tocotrienols effectively inhibit the peroxidation of cell membranes by ROS (Packer et al. 2001) and are likely located in plastid membrane (Munné-Bosch and Alegre 2002). It has been also suggested as the main native antioxidant in raw rubber because of its large quantity and stability in rubber during coagulation and warm air drying steps (Tirimanne et al. 1971). There are 3 tocotrienol isomers in latex, namely α-tocotrienol, γ-tocotrienol and δ-tocotrienol (Dunphy et al. 1965; Whittle et al. 1966; Lee 1993; Yacob et al. 2012). γ-tocotrienol is the most abundant molecular variant in latex and all tocotrienols could amount to about 8% of total lipids (Dunphy et al. 1965; Chow and Draper 1970).

There are 4 rate-limiting enzymes in tocotrienol biosynthesis pathway in plant (Hussain et al. 2013) (Figure 12). Isopentenyl diphosphate (IPP) is the precursor of geranylgeranyl diphosphate. Homogentisate phytyltransferase (VTE2) converts geranylgeranyl diphosphate into 6-geranylgeranyl-2-methylbenzene-1, 4-diol. MPBQ/MSBQ methyltransferase (VTE3) converts 6-geranylgeranyl-2-methylbenzene-1, 4-diol into 6-geranylgeranyl-2, 3-dimethylbenzene-1, 4-diol. Tocopherol cyclase (VTE1) converts 6-geranylgeranyl-2, 3-

dimethylbenzene-1, 4-diol into γ-tocotrienol and 6-geranylgeranyl-2-methylbenzene-1, 4-diol into γ-tocotrienol δ-tocotrienol. Tocopherol γ-methyltransferase (VTE4) converts γ-tocotrienol into α-tocotrienol (Figure 13).

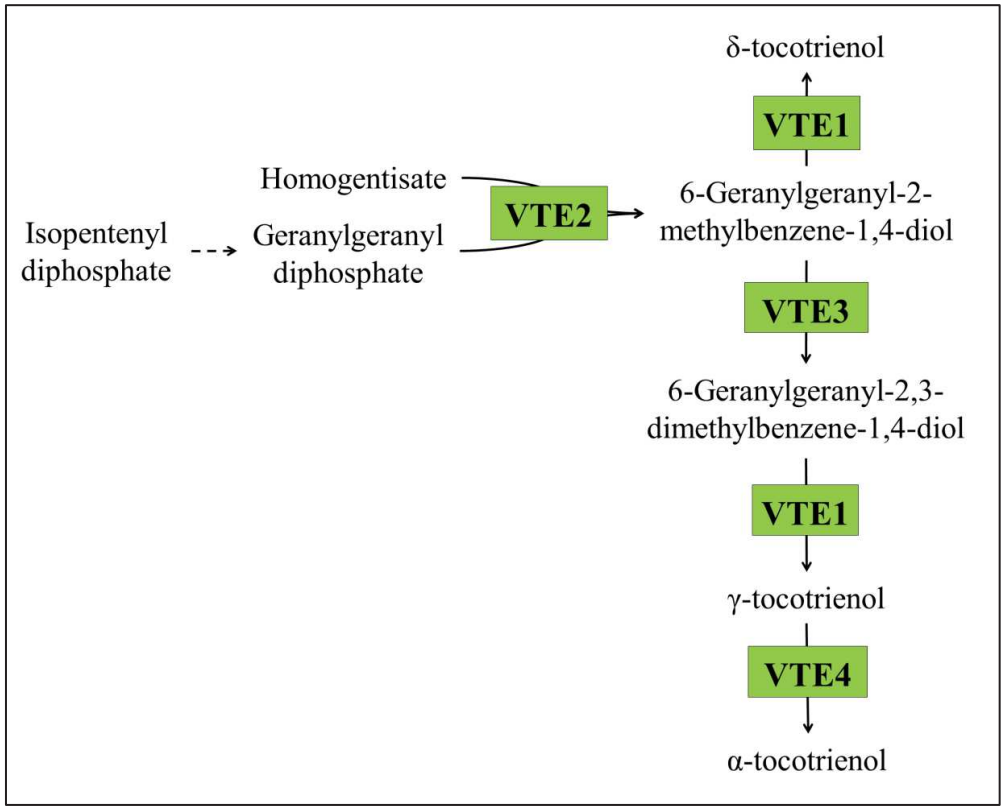

Figure 12. Tocotrienol biosynthesis in plant. Scheme modified from (Hussain et al. 2013)

11. *Hevea* ethylene response factor (ERF) involved in the regulation of redox system

Ethylene releaser is applied widely to stimulate latex production (Figure 7.a). It reveals that ethylene signal pathway play an important role to regulate the latex production. Many biochemical processes related to latex production were regulated by ethylene signal pathway which was documented previously. However over stimulation by ethylene releaser would cause TPD (Putranto et al. 2015b) which indicated ethylene signalling pathway might be involved in regulation of redox system in *Hevea* latex to trigger TPD.

Comprehensive transcriptome analyses led to identify 114 *Hevea* AP2/ERF genes in transcriptome database from *Hevea* clone PB 260 (Duan et al. 2013; Piyatrakul et al. 2014a) which gave an opportunity to study the HbERFs involved in the regulation of redox system. Two ROS-scavenging genes and nine ethylene signaling genes were upreguted by ethylene releaser treatment only in the latex of TPD tree (Putranto et al. 2015b). In plant, RRTF1 (Redox regulation transcription factor 1) has been demonstrated as a master regulator of the redox status (Khandelwal et al. 2008). HbERF-Xb1, unique gene found to be orthologous of RRTF1, were highly accumulated in latex after tapping and ethephon stimulation. HbERF-IXc4 and HbERF-IXc5 genes were confirmed as the *Hevea* orthologues to AtERF1 and functional as activator-

type of transcription factors. AtERF1 is an upstream component in both jasmonate (JA) and ethylene (ET) signaling and is involved in pathogen resistance(Cheng et al. 2013). HbERF-IXc4 and HbERF-IXc5 were synergistically induced by the combination of ET and JA (Putranto et al. 2015a). In addition, their promoters have ARE1 (antioxidant-responsive element 1) *cis*-acting elements revealing their putative involvement in the response to oxidative stress (Piyatrakul et al. 2014a). Indeed, the ARE1 is a regulatory motif of redox sensing mechanisms (Rushmore et al. 1991; Hayes and McMahon 2001).

12. Objective of the PhD

This PhD aims at to identify all redox-related genes, which are involved in ROS-production, regulation and scavenging and main antioxidants biosynthesis in *Hevea* and in particularly those expressed in latex in order to propose a functional model of regulation based on their subcellular localization (sequence analysis and available biochemical information) and on the posttranscriptional regulation by microRNA-targeted transcripts. The whole project started from genome and transcriptome databases, microRNA information (Figure 13). The brown part in Figure 13 represents my contribution to this project and the white part is our prospects. This project was organized as follow:

1. Literature survey of redox system to gather all available information in rubber tree (bibiliographical analysis).
2. Literature survey of redox systems in *Arabidopsis thaliana* with the downloading redox-related proteins sequences.
3. Blast those protein sequences against *Hevea* genome and *Hevea* transcriptome to identify *Hevea* redox-related genes.
4. Annotation of redox-related genes on the rubber tree.
5. Gene expression analysis of redox-related genes in latex from transcriptomic data.
6. Subcellular localization of redox-related proteins in latex by available tools.
7. Post-transcriptional regulation of redox-related genes in latex using degradome data.
8. Identification of target genes of a transcription factor known in Arabidopsis as a master regulator of redox homeostasis by TARGET system.

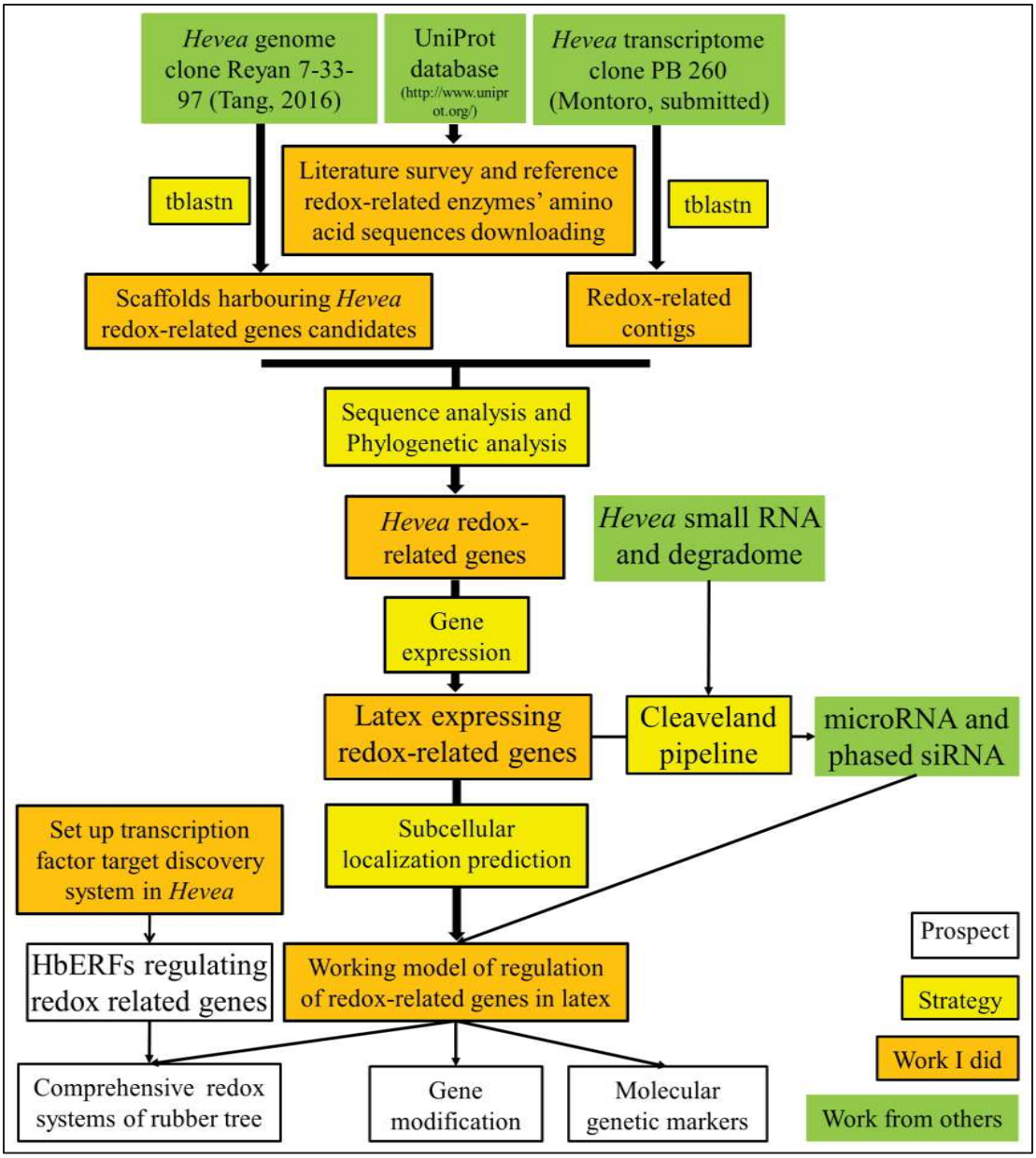

Figure 13. Scheme of general strategy for studying the regulation of network related system in laticifer.

Chapter 1

Reactive oxygen species in *Hevea brasiliensis* latex and relevance to Tapping Panel Dryness

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Introduction

The production and processing of natural rubber had led to many studies on redox reactions and ROS-scavenging systems in laticifers, and on the supply of antioxidants to protect the rubber polymer. To study the redox systems in *Hevea* latex, a comprehensive bibliography study of this topic was needed.

This chapter consists of a review paper gathering the main published information related to ROS-production, ROS-scavenging and main antioxidants in *Hevea* latex. We used several bibiographical libraries (PubMed and Web of Science Core Collection) to collect papers published in national and international journals. Indeed, a large number of papers was published in non-peer reviewed papers and this analysis led to have a chronological order from 1948 to 2016. Including activities of main ROS prodution and scavenging enzymes and main antioxidant molecules, which were studied in those papers. During 1940s to 2000s, researchers were focusing on the physiology and biochemistry studies of redox systems in *Hevea* latex. The main chemical compositions and ROS-production and scavenging enzyme acticities in latex were investigated. With the developments of molecular biology, genomics, transcriptomics and proteomics in *Hevea*, some redox-related genes in latex were studied after 2000.

Production and scavenging of reactive oxygen species in *Hevea brasiliensis* laticifers, a non-photosynthetic tissue

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Running title: ROS and antioxidants in laticifers

Abstract Environmental stress can lead to oxidative stress resulting from an increase in reactive oxygen species (ROS) and involves redox adjustments. Natural rubber is synthesized in laticifers, which are a non-photosynthetic tissue particularly prone to oxidative stress. This paper reviews the current state of knowledge on the regulation of ROS production and ROS-scavenging systems in laticifers. These regulations have been the subject of intense research into a physiological syndrome, called Tapping Panel Dryness (TPD), affecting latex production in *Hevea brasiliensis*. In order to prevent TPD occurrence, monitoring thiol content appeared to be a crucial factor of latex diagnosis. Thiols, ascorbate and γ-tocotrienol are the major antioxidants in latex. They are involved in membrane protection from ROS and likely in quality of raw rubber. Some transcription factors might play a role in the redox regulatory network in *Hevea*, in particular Ethylene Response Factors, which have been the most intensively studied given the role of ethylene on rubber production. Current challenges for rubber research and development with regard to redox systems will involve improving antioxidant capacity using natural genetic variability.

Keywords antioxidant, *Hevea brasiliensis*, latex, reactive oxygen species, rubber tree

Introduction

Oxidation-reduction (redox) reactions involve a transfer of electrons between two compounds. The level of oxidation of a molecule, atom, or ion changes by gaining or losing an electron. Redox reactions are common and vital to some of the basic biological functions such as stress response, development, photosynthesis and respiration (Mittler 2002; You and Chan 2015). Redox homeostasis is necessary to maintain a cell or compartment environment in favour of biological processes. A low level of reactive oxygen species (ROS) generation in the basal redox state of cells or tissues is under the control of a ROS-scavenging system. Abiotic and biotic stress, as well as some plant development processes, are known to trigger disturbances in the basal redox state, which subsequently generates high levels of ROS e.g. $^1O_2$ (singlet oxygen), $O_2^{\cdot -}$ (superoxide radical), $^{\cdot}OH$ (hydroxyl radical) and H_2O_2 (hydrogen peroxide). Peroxides and free radicals damage all components of the cell, including proteins, lipids and nucleic acids. Most of them are very reactive and short lived in cells or tissues. Instead of measuring ROS levels directly, by-products of lipids, proteins, or nucleic acids involved in ROS reactions can be measured to observe oxidative stress indirectly (Shulaev and Oliver 2006). ROS are also involved in plant development such as leaf extension, cell wall stiffening or loosening and root development (Rodriguez et al. 2002; Dunand et al. 2007; Shafi et al. 2015; Karkonen and Kuchitsu 2015). Indeed, ROS are also described as secondary messengers that are perceivable and able to initiate a signal transduction pathway (Baxter et al. 2014a; Foyer and Noctor 2005b). Although not well described, ROS are known to activate the transcription of genes enabling an appropriate response to oxidative stress (Woolley et al. 2013). ROS have very different physical and chemical properties that allow them to modify several types of molecules. The local accumulation of ROS causes post-transcriptional modifications of the cysteine, methionine and histidine residues of proteins that are reversible for nanomolar concentrations or irreversible for millimolar concentrations (Woolley et al. 2013). The oxidation-related changes may change the conformation of proteins or their properties. Transcription factors are targets of redox status changes (Dietz 2014). Some genes inducible by the redox status harbour in their promoter sequences a *cis*-acting element called ARE (Antioxidant Responsive Element) (Garreton et al. 2002).

ROS-scavenging systems play an essential role in maintaining redox homeostasis. Activities of antioxidant enzymes (superoxide dismutase, peroxidase, catalase, and glutathione reductase)

and concentrations of antioxidant molecules (glutathione and ascorbate) are the most predominant functions in plants. Superoxide dismutase (SOD) activity constitutes the first line of defence against ROS by converting $O_2^{\cdot -}$ to H_2O_2. Depending on the metal co-factor used by the enzyme, SODs are classed in three groups: copper-zinc SOD (CuZnSOD), manganese SOD (MnSOD) and iron SOD (FeSOD). In addition, the sub-cellular locations of SODs are different: FeSODs are chloroplastic isoforms, MnSODs are mitochondrial or peroxisomal isoforms and CuZnSODs are cytosolic, chloroplastic or peroxisomal isoforms (Alscher et al. 2002). Hydrogen peroxide is thus converted into H_2O by ascorbate peroxidase (APX), glutathione peroxidase (GPX) and catalase (CAT). Since catalase has a far lower affinity for H_2O_2 (Halliwell 1974), ascorbate peroxidase can scavenge H_2O_2 more efficiently that catalase at low H_2O_2 concentrations. By contrast, catalase is a very potent enzyme at high H_2O_2 concentrations because one molecule of catalase can decompose millions of hydrogen peroxide molecules into oxygen and water. Ascorbate-glutathione cycle steps rely on electrons supplied by reducing compounds of low molecular weight, such as ascorbate and glutathione (Noctor and Foyer 1998a). In the glutathione-ascorbate cycle H_2O_2 is reduced to H_2O by APX and GPX using ascorbate and glutathione as the electron donor respectively. The oxidized ascorbate (monodehydroascorbate) is regenerated by monodehydroascorbate reductase (MDHAR). Given monodehydroascorbate is a radical, it rapidly disproportionates into ascorbate and dehydroascorbate. Dehydroascorbate is reduced to ascorbate by dehydroascorbate reductase at the expense of reduced glutathione (GSH), which is oxidized to form GSSG. The oxidized glutathione can be reduced by glutathione reductase (GR) using NADPH as the electron donor. Thus, ascorbate and glutathione are not consumed and the net electron flow is from NADPH to H_2O_2.

Latex cells amount to a unique cell factory involving redox systems. Among about 2500 latex-producing plant species, *H. brasiliensis* is the main source of natural rubber (NR), which accounts for 42% of total world consumption of rubber. The polymer cis-1,4-polyisoprene, known as natural rubber, is synthesized in the rubber particles of laticifers, which are articulated and anastomosed latex cells (d'Auzac and Jacob 1989; de Faÿ and Jacob 1989). Latex is the cytoplasm of these specialized tube cells. Laticifers are differentiated from vascular cambium (Figure 14 A). The articulated laticiferous vessels are arranged in concentric rings in the phloem (Figure 14 B). Latex flows out from the laticifers without mitochondria after cutting of the soft bark (tapping) (Figure 14 C). For certain rubber clones with a low latex metabolism, application of an ethylene releaser (ethephon) to the bark stimulates latex flow and latex regeneration between two tappings (d'Auzac et al. 1997). Environmental and harvesting stresses, as well as the metabolic activity necessary for latex regeneration between two tappings, are sources of ROS. Over-accumulation of ROS can lead to laticifer dysfunctions such as Tapping Panel Dryness (TPD). TPD halts latex flow (Figure 14 D). The production and processing of natural rubber have led to many studies on redox reactions and ROS-scavenging systems in laticifers, and on the supply of antioxidants to protect the rubber polymer. This paper sets out to review for the first time the production and scavenging of ROS in latex cells with regard to rubber production ROS-associated TPD, and rubber quality. Finally, this paper surveys the inputs of research in terms of genetic improvement and latex diagnosis for monitoring plantations and rubber quality.

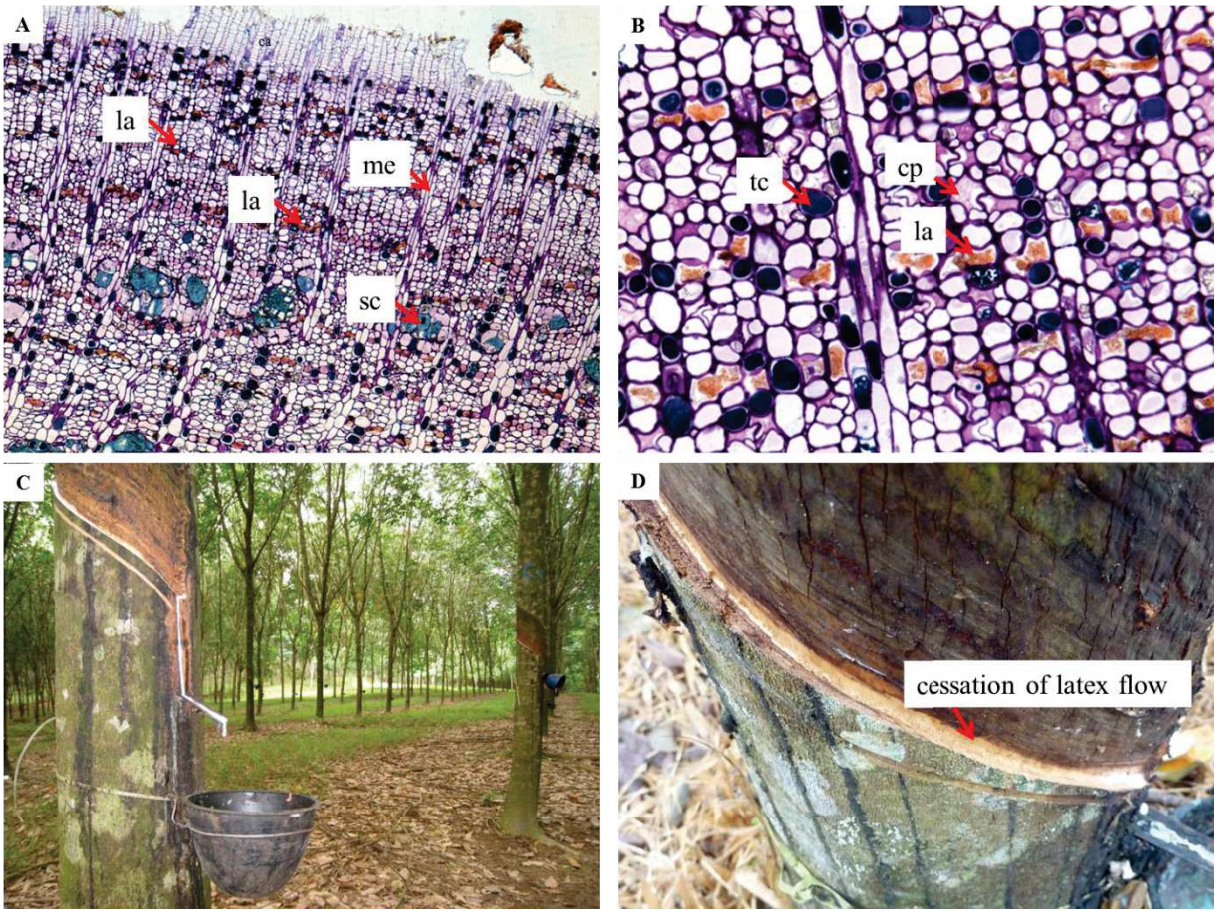

Figure 14. Illustration of laticifer anatomy, latex production and Tapping Panel Dryness (TPD) symptoms. (A) Histological transversal section of phloem tissue (staining with oil-red O and toluidine blue, magnification x5): (la) latex cells are stained in orange-red, (ca) cambium, (me) medullar ray, (cp) conducting phloem, (tc) tannin cell, (sc) sclereid (stone cell). (B) Histological transversal section of laticifer (staining with oil-red O, magnification x20). (C) Normal latex flow after tapping. (D) Partial cessation of latex flow related to TPD.

Regulation of redox systems in latex cells

ROS production and scavenging systems in latex cells have been studied for a long time, and are summarized in Figure 15. The first reported source of ROS in latex was peroxidase (de Haan-Homans 1950a). Then polyphenoloxidase (PPO) (Tata and Edwin 1970), and a specific PPO, *o*-diphenol oxidase (Coupé et al. 1972a), were reported. The main sources of ROS are produced by specific organelles (Table 1). Indeed, latex cells are non-photosynthetic cells harbouring specific compartments such as rubber particles, lutoids and Frey-Wyssling particles (de Faÿ et al. 1989). Frey-Wyssling particles are very specialized chromoplasts. These globules of 0.5 to 2 µm in diameter have a double membrane and contain lipids and carotenoids. These plastids have *o*-diphenol oxidases (ODP), which are a source of ROS (Coupé et al. 1972a). Lutoids are lysosomal micro-vacuoles of 1 to 3 µm in diameter, enclosed by a single membrane. They generally amount to 10 to 20% volume of fresh latex, and have been considered as the major source of ROS in latex cells (d'Auzac et al. 1989). NADH-cytochrome c oxidoreductase activity was first measured in the membrane of isolated lutoids, but surprisingly that extract was not able to oxidize NADPH (Moreau et al. 1975). Lutoid membranous NADH-cytochrome *c*-reductase was also evolved in an NADH-O_2 reductase, a generator of superoxide ions

(d'Auzac et al. 1982a). Enzymatic activity generating superoxide anions from NAD(P)H and O_2 was later observed (Cretin and Bangratz 1983). Lutoidic NAD(P)H oxidase generates species of toxic oxygen, which lead to peroxidatic degradation of the unsaturated lipids of the membrane (Chrestin et al. 1984). NAD(P)H oxidase was reported as the main ROS source in laticifers, especially when the laticifers were under stress (Cretin and Bangratz 1983; Chrestin et al. 1984).

Table 1. Reactive oxygen species production and scavenging in the latex of Hevea brasiliensis.

Function	Subcellular localization	Evidence level	Reference
ROS production			
Polyphenol oxidase	Cytosol, B-serum	Enzyme activity	(Tata and Edwin 1970)
	Unknown	Protein	(Wang et al. 2015a)
O-diphenol oxidase	Frey-Wyssling particles	Enzyme activity	(Coupé et al. 1972a)
NADPH oxidase	Lutoid membrane	Enzyme activity	(Chrestin et al. 1984)
Peroxidase	Lutoids, cytosol	Enzyme activity	(de Haan-Homans 1950a) (Tata and Edwin 1970; Coupé et al. 1972a; Wititsuwannakul et al. 1997) (Jacob et al. 1984)
	Unknown	Protein	(Wang et al. 2015a)
ROS-scavenging			
Catalase	Cytosol, B-serum	Enzyme activity	(de Haan-Homans 1950a) (Tata and Edwin 1970; Coupé et al. 1972a) (Jacob et al. 1984)
Superoxide dismutase	Cytosol, B-serum	Enzyme activity	(Jacob et al. 1984)
	Cytosol	Enzyme activity	(Clément et al. 2001)
	Cytosol	Protein	(Jiyan 2011)
	Unknown	Protein	(Wang et al. 2015a)
	Unknown	mRNA	(JinQuan et al. 2015)
	Cytosol	Transgenic plant	(Leclercq et al. 2012)
Ascorbate peroxidase	Cytosol	Enzyme activity	(Clément et al. 2001)
	Unknown	Protein	(Wang et al. 2015a)
	Unknown	mRNA	(JinQuan et al. 2015)Putranto 2014)
	Cytosol	mRNA	(Chao et al. 2015b)
Monohydroascorbate reductase	Unknown	Protein	(Wang et al. 2015a)
Dehydroascorbate reductase	Unknown	Enzyme activity	(Clément et al. 2001)
	Unknown	Protein	(Wang et al. 2015a)
Glutathione peroxidase	Cytosol	Enzyme activity	(Jacob et al. 1984)
	Cytosol	Enzyme activity	(Clément et al. 2001)
	Unknown	mRNA	(Yujie 2011)
Glutathione reductase	Cytosol	Enzyme activity	(Prevot et al. 1984a) (Jacob et al. 1984)
	Cytosol	mRNA	(Deng et al. 2014)
Glutathione S-transferase	Unknown	Enzyme activity	(Balabaskaran and Muniandy 1984)
	Unknown	Protein	(Wang et al. 2015a)
Ascorbate	Cytosol	1.1 mM	(Archer 1969)
Glutathione	Cytosol	0.3 mM	(Archer 1969)
Tocopherol/tocotrienol	Membrane	8% of lipids	(Dunphy et al. 1965)
Ascorbate biosynthesis			
GDP-L-galactose phosphorylase	Unknown	mRNA	(Yujie 2011; Tang et al. 2013b)
GDP-mannose-3′,5′ epimerase	Unknown	mRNA	(Tang et al. 2013b)
Tocopherol/tocotrienol biosynthesis			
Geranylgeranyl reductase	Unknown	Protein	(Wang et al. 2015a)

Redox homeostasis is controlled by the biosynthesis and reduction of antioxidants and by ROS-scavenging enzymes. Latex contains three major antioxidants, namely thiol, ascorbate and tocotrienol. Some other molecules with antioxidant powers can be also detected, such as

phytosterols, phospholipids, phenols, betaines, proteins and amino acids. The total thiol concentration is above 0.5 to 0.9 mM in latex (Jacob et al. 1984), and can reach up to 2.2 mM (Chrestin 1984). Up to 90% of them are glutathione and cysteine (McMullen 1960). Cysteine is an important biochemical precursor for glutathione synthesis (Franklin et al. 2009). The concentration ratio between glutathione and cysteine in latex is 1.6 : 1 (McMullen 1960). The concentration of ascorbate can range from 1.9 to 3.9 mM (Archer 1969; Chrestin 1984). There are 4 vitamin E isomers in latex, namely α-tocopherol, α-tocotrienol, γ-tocotrienol and δ-tocotrienol (Dunphy et al. 1965; Lee 1993; Whittle et al. 1966; Yacob et al. 2012). α-tocopherol is the saturated isoform of tocotrienols. γ-tocotrienol is the most abundant molecular variant in latex and all tocotrienols could amount to about 8% of total lipids (Dunphy et al. 1965; Chow and Draper 1970).

The ascorbate and glutathione biosynthesis pathways have been partially characterized (Fan 2011; Putranto et al. 2012). D-mannose/L-galactose pathway is the most significant source of ascorbate in plants. GDP-L-Galactose phosphorylases and GDP-D-mannose-3',5'-epimerase are important enzymes related to this pathway (Ishikawa and Shigeoka 2008). Two genes encoding GDP-L-galactose phosphorylases were upregulated during the first 5 tappings of re-opened rubber trees in this pathway (Fan 2011). Interestingly, one gene encoding a GDP-D-mannose-3',5'-epimerase was expressed at a higher level in a super-high-yielding tree (Tang et al. 2013a). This super-high-yielding tree is more capable of lowering stress levels over time, thereby making it possible to invest more effort in the metabolic pathways related to latex regeneration. The antioxidant power of glutathione and ascorbate is also intensively regenerated by the enzymes of the glutathione-ascorbate cycle. Dehydroascorbate reductase, glutathione reductase (Jacob et al. 1984; Prevot et al. 1984a), cytosolic glutathione reductase (Deng et al. 2014), ascorbate peroxidase, and at least two glutathione peroxidases have been characterized (Clément et al. 2001; Dai et al. 2013a). A gene encoding a glutathione peroxidase was upregulated during the first 5 tappings of re-opened rubber trees (Fan 2011). An ascorbate peroxidase gene was upregulated in rubber clone CATAS8-79 in which latex regeneration was more effective than in clone PR107 (Chao et al. 2015a). The available NADPH content and the presence of certain inhibitors *in situ*, such as quinoid type molecules, Cu^{2+} and Zn^{2+}, are likely to control glutathione reductase (GR) activity physiologically (Jacob et al. 1984). GR activity was shown 10 times higher in latex than in lutoid (Prevot et al. 1984a). More recently, two *GR* genes were characterized (Deng et al. 2014; Deng et al. 2015). *GR1* and *GR2* genes are expressed in latex and induced by ethylene, jasmonate, hydrogene peroxide and wounding treatment. Antioxidant defence enzymes, such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX) and glutathione-S-transferase (GST), are crucial for breaking down the harmful end-products of oxidative phosphorylation. Concomitant with an increase in respiration, tapped trees also enhanced the ROS-scavenging system in soft bark tissues (Annamalainathan et al. 2001).

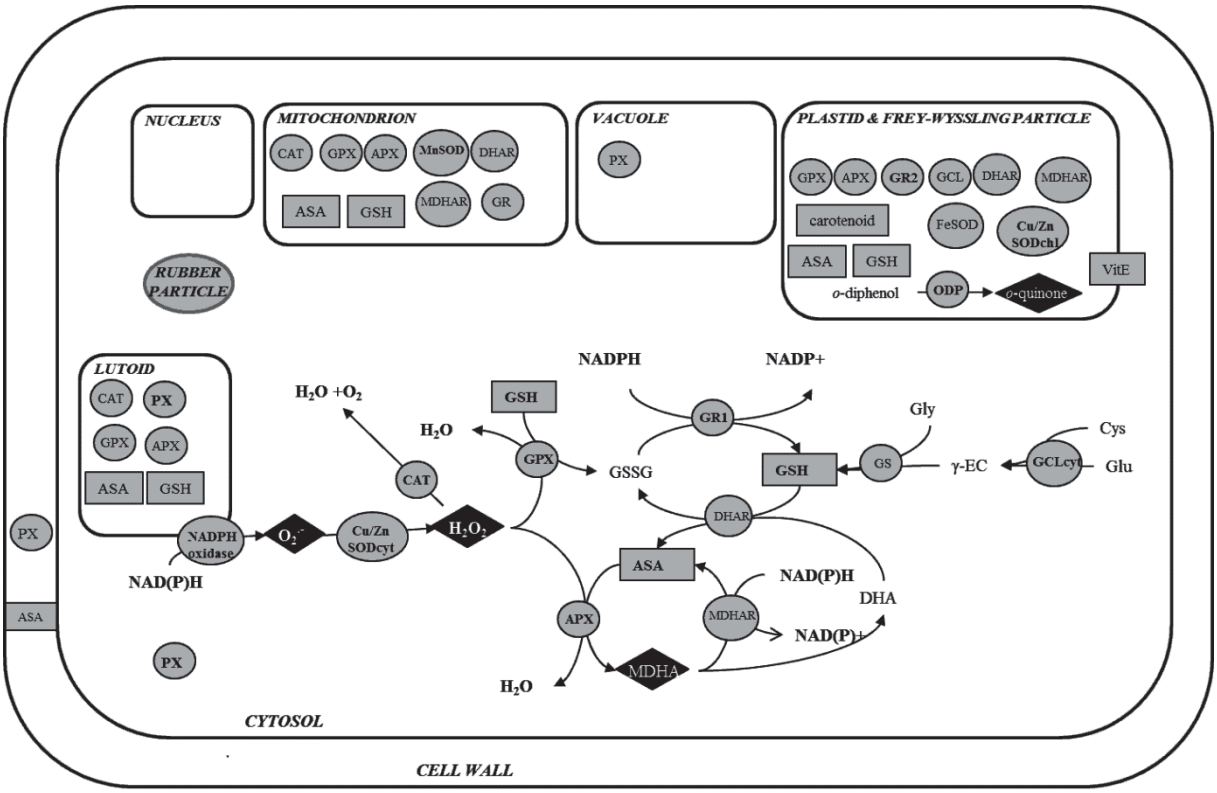

Figure 15. General scheme of ROS production and scavenging systems in latex cells. Enzymes are in grey circle, antioxidants in grey square, and ROS in black diamond. Subcellular localization of enzymes and compounds is specified in normal letter according to Alscher et al. (2002), and in bold letter when experimentally determined. CAT, catalase; PX, peroxidase; ASA, ascorbate; GSH, glutathione; APX, ascorbate peroxidase; GPX, glutathione peroxidase; MDHAR, monodehydroascorbate reductase; DHAR, dehydroascorbate reductase; GR, glutathione reductase; GCL, glutamate cysteine ligase; GS, glutathione synthetase; Gly, glycine; γ-EC, γ-glutamylcysteine; Cys, L-cysteine; Glu, L-glutamate; ODP, *o*-diphenol oxidase. The 4 vitamin E isoforms, namely α-tocopherol, α-tocotrienol, γ-tocotrienol and δ-tocotrienol, are specified as VitE, and they are assumed to be present in plastid membrane according to Munne-Bosch and Alegre (2002).

Catalase and peroxidase activities were investigated in latex (de Haan-Homans 1950a; Tata and Edwin 1970). 60% to 80% of peroxidase activity was localized in lutoids and the rest in cytosol. 50% of catalase activity was localized in some kind of particle (probably lutoids) and the rest in cytosol (Coupé et al. 1972a). Peroxidases were also investigated in bark of rubber tree (Tian et al. 2015; Wititsuwannakul et al. 1997). Considering the low affinity for H_2O_2 of catalase which may only act to remove high H_2O_2 concentrations, in case of oxidative burst. APX and GPX activities, with high affinity, are suitable for detoxification of low amounts of H_2O_2 (Clément et al. 2001). Recently, the down-regulation of a *HbAPX* gene by ethephon was suggested to disturb the redox homeostasis in laticifer cells of rubber tree (Chao et al. 2015c).

Superoxide dismutase activity was first reported by d'Auzac (d'Auzac et al. 1989). This enzyme is encoded by a multigene family consisting of a Mn superoxide dismutase (Miao and Gaynor 1993) and two Cu/Zn superoxide dismutases, a cytosolic isoform (Leclercq et al. 2012), and a chloroplastic form (Gébelin et al. 2013a). The *MnSOD* gene was first upregulated and then downregulated in latex during the first 5 tappings of re-opened rubber trees (Jiyan 2011).

Interestingly, a superoxide dismutase gene was up-regulated in rubber clone CATAS8-79, in which latex regeneration was more effective than in PR107 (Chao et al. 2015a). The catalase gene was first cloned by Kongsawadworakul and coll. (Kongsawadworakul et al. 1997). Several other redox-related genes have been identified: thioredoxin H type (Chow et al. 2007), hydrogen peroxide-induced metallothionein (HbMT2) (Zhu et al. 2010), thioredoxin and two amine oxidases (Fan 2011). Lastly, a detoxifying enzyme, glutathione S-transferase, was detected in a variety of tissues with a broad pH optimum between 8.5 and 9.5 (Balabaskaran and Muniandy 1984).

ROS-associated TPD affects latex production

Tapping Panel Dryness seriously affects the latex production of a rubber tree plantation. TPD refers to two syndromes (Putranto et al. 2015b). The first is related to overproduction of ROS and consequent cellular damage that can be reversible after resting trees without tapping (Das et al. 2002). The second form, called brown bast, involves histological changes and senescence mechanisms (Li et al. 2015). TPD susceptibility depends on genetic and environmental factors. Overexploitation of rubber trees including a high tapping frequency and ethephon stimulation can cause early TPD occurrence associated with a decrease in thiol content (Putranto et al. 2015b).

ROS generation and subsequent peroxidation of the cellular membrane system were first reported to be involved in latex flow stoppage by Cretin and Bangratz (Cretin and Bangratz 1983). High NAD(P)H oxidase activity at the surface of lutoids was considered as the main source of ROS leading to peroxidative degradation of the unsaturated lipids of the lutoid membranes, then the release of factors involved in latex coagulation (Chrestin et al. 1984). Quinoid type molecules and Cu^{2+} are activators of NADPH oxidase (Chrestin 1989c). Quinoid type molecules, such as plastoquinone and ubiquinol, are components of lutoids (Archer 1969). The concentration of Cu^{2+} in lutoids is twice the concentration in cytosol (d'Auzac et al. 1982a). The quinoid type molecules and Cu^{2+} released from lutoids at the beginning of lutoid bursting probably inhibit glutathione reductase activity but activate NADPH oxidase activity. In other words, ROS accumulation enhances the peroxidative degradation of lutoid membranes, which is a positive feedback to lutoid bursting. In a second step, *o*-diphenol oxidase activity specifically expressed in Frey-Wyssling particles was noted in cytosol from TPD-affected trees revealing the lysis of Frey-Wyssling particles (Cretin and Bangratz 1983). Hevein was then shown to be involved in the agglutination of rubber particles (Gidrol et al. 1994b). Another *Hevea* latex lectin-like protein present on the lutoid membrane, the small rubber particle protein (SRPP), was reported to induce aggregation of rubber particles and lutoid membranes (Wititsuwannakul et al. 2008b).

Typical TPD symptoms exhibit abnormally high NAD(P)H oxidase and peroxidase activities, but also a very low activity in ROS-scavenging enzymes such as SOD and CAT (Chrestin 1989b). This was confirmed on the bark of trees overstimulated with a high concentration of ethephon, which can generate higher concentrations of free radicals and exhibit lower SOD activity than in an untreated tree (Das et al. 1998). SOD and glutathione s-transferase protein contents decreased in latex after ethephon stimulation (Wang et al. 2015b). Taken together with the protein accumulation of peroxidase and monodehydroascorbate peroxidase in ethephon stimulated tree (Wang et al. 2015b), this indicates that a high ethephon concentration is a ROS-related toxin for latex tissue. The expression of *CAT* and *Mn-SOD* genes can be stimulated by moderate ethylene treatment in a healthy tree but not in trees affected by TPD (Kongsawadworakul et al. 1997). By contrast *GR1* and *GR2* genes are upregulated in latex and bark of TPD-affected trees (Deng et al. 2014; Deng et al. 2015). Some other ROS-scavenging

systems have been identified but not clearly characterized. For instance, inhibitors of NAD(P)H-quinone-reductase activity were suggested to be either directly involved in this enzyme inhibition or indirectly, by scavenging toxic oxygen produced by the reaction; the possibility of using these inhibitors *in situ* on the tapping panel was suggested (d'Auzac et al. 1986). Generally speaking, antioxidants and ROS-scavenging enzymes are related to the preservation of rubber production capacity (Lacote et al. 1998; Das et al. 2002).

Over the last decade a substantial effort has been made in understanding transcriptional regulation when TPD occurs. Expression of the *HbMyb1* transcription factor was significantly decreased in the barks of TPD trees (Chen et al. 2003). In another report, down-regulation of another Myb transcription factor and the thioredoxin H-type gene was shown in TPD trees (Venkatachalam et al. 2007). The suppression of stress-induced cell death by HbMyb1 was demonstrated in transgenic tobacco (Peng et al. 2011). Recent development of Next-Generation Sequencing technology has made it possible to identify both small RNAs and transcripts differentially expressed in trees affected by TPD (Gébelin et al. 2013b; Liu et al. 2015). According to the Gene Ontology annotations, 20 miRNA families are involved in regulating the expression of antioxidant activity genes (Gébelin et al. 2012). About 70 antioxidant activity genes were expressed in the bark of healthy and TPD-affected trees (Mantello et al. 2014; Liu et al. 2015). However, only 7 antioxidant activity genes were predicted in latex (Wei et al. 2015c).

Is thiol content a key factor of latex diagnosis?

The study of natural rubber-producing laticifer systems led to the development of latex diagnosis measuring sucrose, inorganic phosphorus, total thiols and dry rubber contents. These parameters are positively correlated with latex production (Eschbach et al. 1984; Prevot et al. 1984b; Sreelatha et al. 2009). Latex diagnosis helps in optimizing harvesting systems, tapping frequency and ethephon stimulation, in order to avoid any risk of TPD. The total reduced thiol content is one of these latex diagnosis parameters. Thiols are organosulphur compounds that contain a carbon-bonded sulphydryl (–C–SH or R–SH) group, where R represents an alkane, alkene, or other carbon-containing group of atoms. Total thiols provide a powerful reductive pool in latex (McMullen 1960). Harvesting stresses are known to activate latex metabolism and lead to R-SH consumption in latex (Krishnakumar et al. 2009). The thiol content of latex is subject to variation after hormone treatments and depends on the clonal origin of latex (Eschbach et al. 1984). In addition, the thiol content is subject to variation due to the environment. A negative correlation between thiol concentration and latex yield has been reported for rubber trees growing at low temperatures (Alam et al. 2003). In the event of overexploitation, a drop in thiol concentration was always observed in the final phase of rubber harvesting, while a variable concentration might be measured in the first phase (Jacob et al. 1989a). Consequently, the identification of a more consistent antioxidant parameter than reduced thiols should be useful for monitoring rubber plantations through latex diagnosis, as well as developing high throughput phenotyping of segregating populations in breeding programmes for the selection of high-antioxidant clones.

Vitamin E hampers oxidation during raw rubber processing

Natural antioxidants in latex are involved in the quality of natural rubber in fresh harvested latex, and during rubber maturation and processing. Oxidative degradation occurs during storage hardening of raw rubber (Morris 1991). Natural antioxidants might hamper such oxidation but are not sufficient in latex to protect the polymer. *Cis*-1,4-polyisoprene with its

high level of unsaturation is particularly prone to oxidative degradation. Latex antioxidants hamper such oxidation but are not sufficient to protect the polymer in the rubber products. Natural and synthetic commercial antioxidants are used as an aging retardant in natural rubber vulcanizates (Hossain et al. 2010; Komethi et al. 2012). Among normal oxidative facilitating factors, such as heat, light, presence of prooxidants and mechanical strain, heat effect is the most significant factor to accelerate the oxidative aging (Mathew and De 1983). Raw rubber processing, from fresh latex to dry raw rubber, contains an important step of drying in which rubber is bathed in a warm air of around 100 °C. Thanks to the protection of natural antioxidants from latex, it is not necessary to add stabilizers in raw rubber during the drying step as in the case of synthetic rubber (Tirimanne et al. 1971). Vitamin E, phytosterols, phospholipids, phenols, betaines, proteins and some amino acids from the latex can act as antioxidants against oxidation in raw rubber (Altman 1948; Dunphy et al. 1965; Tirimanne et al. 1971; Musigamart et al. 2014). Among the latex antioxidants, vitamin E has been suggested as the main native antioxidant in raw rubber because of its large quantity and stability in rubber during coagulation and warm air drying steps (Tirimanne et al. 1971). The fat-soluble ability of vitamin E can help it to persist in raw rubber during processing (Liengprayoon et al. 2013), and it maintains antioxidant potency *in vitro* (Kamal-Eldin and Appelqvist 1996). Analysing the dynamic of tocotrienol was even suggested as the resistance parameter of rubber to oxidation during the raw rubber processing (Musigamart et al. 2014).

Towards a redox regulatory network in *Hevea*

Characterization of the Ethylene Response Factor (ERF) gene family in *Hevea* has led to the identification of several ERFs putatively involved in the regulation of redox genes (Piyatrakul et al. 2014b). Their regulation by harvesting stress and their putative orthologs in *Arabidopsis* are presented in Figure 16. *HbERF-Xb1* gene is orthologous to *RRTF1*, which has been described as the main node of the redox responsive co-expression network that controls a regulon responsive to a change in redox status (Khandelwal et al. 2008). Another ERF, RAP2.4a, was the first redox-modified transcription factor to be identified. This protein adopts conformational change according to the redox status. It binds to the target promoter of the *2CPA gene as a dimer only under physiological redox conditions. Otherwise, under* reducing conditions and oxidizing conditions, the inactive transcription factor stays as a monomer or an oligomer, respectively (Shaikhali et al. 2008). This gene should belong to *Hevea* ERF group Ia (Piyatrakul et al. 2014b), but to date there are no identified orthologs in the *Hevea* transcriptome. The new complete genome version is expected to provide additional genes that could include this gene.

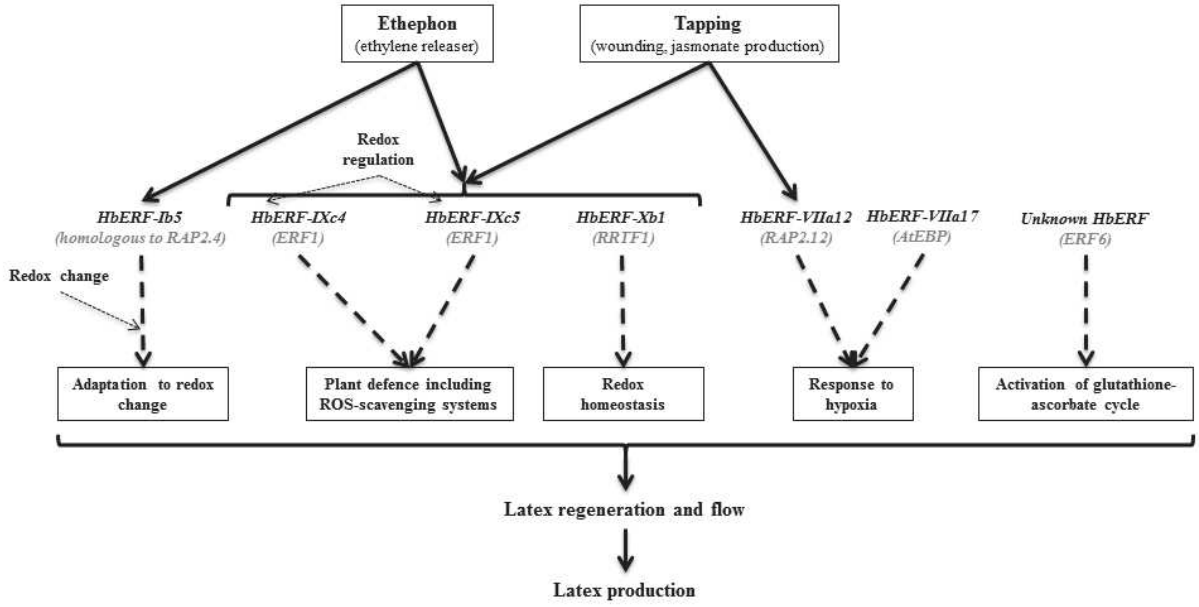

Figure 16. Working model of the regulatory network controlling redox systems and response to hypoxia in Hevea through Ethylene Response Factors. Black arrow: activation of function. Dotted arrow: assumption based on function demonstrated in Arabidopsis. Grey letter: ortholog gene in Arabidopsis based on phylogenetic analysis. Promoters of HbERF-IXc4 and HbERF-IXc5 genes harboured Antioxidant Responsive Elements (ARE) suggesting redox regulation of their transcription.

The biosynthesis of antioxidant compounds is also greatly controlled by ERF transcription factors. To date, no orthologous gene has been identified in rubber (Piyatrakul et al. 2014b). In *Arabidopsis*, ERF98 activates the genes involved in the ascorbate biosynthesis pathway (Zhang et al. 2012). Some ROS-inducible ERFs have also been described in *Arabidopsis*. ERF6 is probably indirectly an activator of genes involved in the glutathione-ascorbate cycle, such as *DHAR1*, *APX4* and *CAT1*, because there is no GCC-box in the promoter of these target genes (Sewelam et al. 2013). Only promoters of two *ERF* genes, *HbERF-IXc4* and *HbERF-IXc5*, harbour an ARE *cis*-acting element revealing the putative response to the redox status of these genes (Piyatrakul et al. 2014b; Putranto et al. 2015a). Although these two transcription factors are orthologs to ERF1, which controls a large panel of defence genes, there is no evidence for the activation of genes encoding ROS-scavenging enzymes (Piyatrakul et al. 2014b).

Oxidative stress is induced by a wide range of environmental factors such as oxygen shortage. Generation of ROS in mitochondria was observed for hypoxia and especially for reoxygenation. In TPD-affected trees, the consumption of oxygen by NADH-cytochrome-c-oxidoreductase was particularly high and hypoxia condition was observed (Chrestin 1989b). *HbERF-VIIa12* and *HbERF-VIIa17* genes are putative orthologs to *RAP2.12* and *AtEBP*, which are involved in the activation of hypoxia-responsive genes through the N-end rule pathway (Piyatrakul et al. 2014b). AtEBP also confers resistance to hydrogen peroxide and heat treatments (Gibbs et al. 2011). *HbERF-VIIa12* and *HbERF-VIIa17* genes are induced by tapping and constitutively highly expressed in latex, respectively, and might play a role in hypoxia response.

The genes involved in the ROS-scavenging system are also subjected to microRNA-mediated post-transcriptional regulations. Small RNAs have been deeply sequenced in *Hevea* in various

plant tissues and in the latex of healthy and TPD-affected trees (Gébelin et al. 2012; Gébelin et al. 2013b; Lertpanyasampatha et al. 2012). Several ROS-scavenging enzymes have been identified as targets of these microRNAs. The cleavage site by Hbmir398 has been experimentally validated for the chloroplastic CuZnSOD isoform only (Gébelin et al. 2012), and regulates the mRNA level of its target gene in response to salinity (Gébelin et al. 2013a). *Rboh* transcripts have been predicted to be targeted by two miRNAs (HbmiR2914 and HbmiR476) (Gébelin et al. 2012).

Challenges for rubber research and development with regard to redox systems

This paper reviewed literature on the production and scavenging of ROS in latex cells and revealed that redox reactions are key functions for natural rubber production and quality, as well as tolerance of biotic and abiotic stress. Several transcriptomic analyses showed transcriptional regulation of redox genes but we are far away from a comprehensive understanding of the regulation brought into play. The functional analysis of redox systems will necessitate an integration of proteomic and metabolomic information. This approach could lead to the identification of new factors, such as monoterpene which might be a very effective molecule in protecting rubber plants against oxidative stress (Chen et al. 2009). A role in the protection of raw rubber against thermo-oxidation has also been suggested for vitamin E. Given the large amount of vitamin E, and especially tocotrienol, these compounds could be exploited from waste serum generated during the processing of deproteinised natural rubber (Sajari et al. 2014).

Successful attempts have been made to engineer rubber plants with a high antioxidant capacity. Transgenic plants over-expressing *HbMnSOD*, cytosolic *HbCuZnSOD* and *EcGSH1* have been regenerated and characterized (Jayashree et al. 2003; Leclercq et al. 2012; Martin et al. 2015). Over-expression of the *HbCuZnSOD* and *EcGSH1* genes resulted in the production of fast-growing plants with greater tolerance of abiotic stress. Interestingly, these authors showed only that cytosolic *HbCuZnSOD* genes had no post-transcriptional regulation by microRNA398 which could affect the expression of these transgenes (Gébelin et al. 2012; Leclercq et al. 2012). As regards glutathione biosynthesis, the two *Hevea* genes encoding the glutamyl cysteine ligase (GCL) are targeted by a microRNA but not the bacterial gene (*EcGSH1*) used in the experiment (Gébelin et al. 2013a). These transgenic plants accumulated 3 times more glutathione than wild-type plant material (Martin et al. 2015). Further applications of genetic engineering need to deal with the concerns of the public and NR supply chains regarding GMO dissemination (Smith 2011). This social debate should encourage researchers to use genetic variability in *Hevea* germplasm to improve tolerance of ROS-induced TPD and abiotic stress through conventional breeding programmes.

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Conclusion

This paper reviewed literature on the production and scavenging of ROS in latex cells and revealed that redox reactions are key functions for NR production and quality, as well as tolerance of biotic and abiotic stress. Several transcriptomic analyses showed transcriptional regulation of redox genes but we are far away from a comprehensive understanding of the regulation brought into play. The functional analysis of redox systems will necessitate an integration of proteomic and metabolomic information. This approach could lead to the identification of new factors, such as monoterpene, which might be a very effective molecule in protecting rubber plants against oxidative stress (JunWen et al. 2009). A role in the protection of raw rubber against thermo-oxidation has also been suggested for vitamin E. Given the large amount of vitamin E, and especially tocotrienol, these compounds could be exploited from waste serum generated during the processing of deproteinized NR (Sajari et al. 2014). Successful attempts have been made to engineer rubber plants with a high antioxidant capacity. Transgenic plants over-expressing *HbMnSOD*, cytosolic *HbCuZnSOD* and *EcGSH1* have been regenerated and characterized (Jayashree et al. 2003, Leclercq et al. 2012, Martin et al. 2015). Overexpression of the *HbCuZnSOD* and *EcGSH1* genes resulted in the production of fast-growing plants with greater tolerance of abiotic stress. Interestingly, these authors showed only that cytosolic *HbCuZnSOD* genes had no post-transcriptional regulation by microRNA398, which could affect the expression of these transgenes (Gébelin et al. 2012, Leclercq et al. 2012). These transgenic plants accumulated three times more glutathione than wild-type plant material (Martin et al. 2015). Further applications of genetic engineering need to deal with the concerns of the public and NR supply chains regarding genetically modified organism (GMO) dissemination (Smith 2011). The public concern about GMOs should encourage researchers to use genetic variability in *Hevea* germplasm to improve tolerance of ROS-induced TPD and abiotic stress through conventional breeding programmes.

Both of figuring out the molecular mechanism of ROS production and scavenging in *Hevea* latex and dealing with TPD by applications of genetic engineering should be based on a well understanding of the information of the genes involved in latex ROS production and scavenging. Then, a work of "Genome-wide analysis of genes involved in production and scavenging of ROS and antioxidant biosynthesis in *Hevea brasiliensis* laticifers" was carried out.

Chapter 2

Genome-wide analysis of genes involved in production and scavenging of reactive oxygen species and antioxidant biosynthesis in *Hevea brasiliensis* laticifers

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Introduction

Base on the comprehensive bibliography study of redox systems in *Hevea* latex, we found there were only physiological and biochemical data investigated and a few redox related genes cloned in latex in the past. And the expressions of some redox-related genes were reported by RNAseq in latex. The comparison of two contrasting clones for latex yield showed that antioxidant-related genes are crucial in the regulation of latex regeneration and duration of latex flow (Chao et al. 2015a). Latex of self-rooted juvenile clones from somatic embryogenesis showed more differential expressed genes (DEGs) related to ROS-scavenging metabolism (Li et al. 2016b). Transcriptomics analysis of a set of rubber clones showed that three and six overexpressed DEGs were involved in ROS production and ROS-scavenging, respectively (Tang et al. 2016). Although all these genes were expressed in latex, several other studies did not report any significant changes in expression of antioxidant genes in latex (Li et al. 2015; Wei et al. 2015a). All these studies globally analysed the gene expression but did not specifically check redox-related gene families. Study on *Hevea* redox systems was very poor.

Now, the availability of *Hevea* genome sequence and high throughput gene expression analysis open the perspective to identify all genes involved in ROS-production, regulation and scavenging and main anitoxidants biosynthesis in *Hevea* and their expression in latex. We did a bibliography study firstly on *Arabidopsis* redox-related genes (genes involved in ROS-production, regulation and scavenging and anitoxidants biosynthesis). We downloaded their amino acid sequences from UniProt database and made tblastn against to the *Hevea* genome, which was provided by Pr. Tang Chaorong (Tang et al. 2016), and transcripstome (Montoro 2017) by the help of two bioinformaticians (Stéphanie Pointet and Enrique Ortega-Abboud) and *via* Galaxy (http://galaxy.southgreen.fr/galaxy/). The tblastn results generated thousands of redox-related sequences. After complete open reading frames (ORF) identification *via* blastx on NCBI one by one of those redox-related sequences, the phylogenetic analysis of those ORF validated genes was carried out *via* Mega 6 with their amino acid sequences. Finally, the *Hevea* redox-related genes were identified and their expression in latex was investigated in this study.

To study the redox systems in latex comprehensively, the subcellular localization of the latex expressing redox-related proteins were predicted *via* three tools (WoLF PSORT, CELLO2GO and Plant-mPLoc). Additionally, microRNAs targeting the latex expressing redox-related genes were predicted by Shuangyang Wu and Julie Leclercq *via* Cleaveland pipeline with the *Hevea* degradome database and small RNA database analysis. The latex expressing redox-related genes' data was completed with targeting microRNA and gene expression analysis by RNAseq of a TPD susceptible *Hevea* clone under various latex harvesting conditions and affected by the Tapping Panel Dryness (Montoro 2017). This study will provide insight into the transcriptional and posttranscritionnal coordination of the oxidant-antioxidant system in relation to the tolerance to latex harvesting stress and TPD.

Genome-wide analysis of redox-related genes in *Hevea brasiliensis* laticifers revealed high and species-specific posttranscriptional regulation

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Running title: ROS production and scavenging genes in laticifers

Abstract

In laticifers, a non-photosynthetic tissue, reactive oxygen species ROS can affect the production and quality of natural rubber. ROS-scavenging enzymes and antioxidants have been biochemically quantified in the latex of *Hevea brasiliensis*. Oxidative stress has been related to latex flow stoppage and consequent loss of natural rubber production. This physiological syndrome, called Tapping Panel Dryness, involves some transcriptional regulations of redox-related genes in latex. This study took advantage of the availability of the rubber genome sequence and transcriptomics data to implement a genome-wide analysis of genes involved in the production and scavenging of ROS and antioxidant biosynthesis. The *Hevea* genome harbours 407 genes of the thirty main redox-related gene families. There are 161 redox-related genes expressed in latex. Thirteen of these redox-related genes were targeted by 12 microRNAs and 15 genes by 16 phased siRNA. A working model was proposed for transcriptional and post-transcriptional regulation with respect to the predicted subcellular localization of deduced proteins. This paper reports on the most complete classification of redox-related genes for a crop species, and reveals a larger number of genes than in *Arabidopsis* and specificity compared to photosynthetic tissues.

Keywords: ethylene, gene expression, latex, microRNA, post-transcriptional regulation, redox, RNA sequencing, ROS, rubber, Tapping Panel Dryness

Introduction

Plant cell redox homeostasis is necessary to promote biological processes (Trchounian et al. 2016). Abiotic and biotic stress, as well as some plant development processes, are known to trigger disturbances in the basal redox state (Rodriguez et al. 2002; Dunand et al. 2007; Shafi et al. 2015; Karkonen and Kuchitsu 2015), which subsequently generates high levels of reactive

oxygen species (ROS) such as $^1O_2$ (singlet oxygen), $O_2^{\circ-}$ (superoxide radical), $^{\circ}OH$ (hydroxyl radical) and H_2O_2 (hydrogen peroxide). Peroxides and free radicals damage all cellular components including proteins, lipids and nucleic acids. ROS are also described as secondary messengers that are perceivable and able to initiate adaptive mechanism (Baxter et al. 2014a; Foyer and Noctor 2005b).

ROS production and scavenging are key functions in the production and quality of natural rubber, as well as tolerance of biotic and abiotic stress in *Hevea brasiliensis* (Zhang et al. 2016b). The rubber tree is the main commercial source of natural rubber, which accounts for about 40% of rubber consumption worldwide. Rubber, the *cis*-1,4-polyisoprene polymer, is synthesized in the rubber particles of laticifers (de Faÿ and Jacob 1989). Latex is the cytoplasm of these articulated laticiferous vessels arranged in concentric rings in the phloem tissue. Latex flows out after cutting the soft bark (tapping). The application of ethephon, an ethylene releaser, to the bark stimulates latex flow and latex regeneration between two tappings (d'Auzac et al. 1997). ROS production takes place in laticifers in response to harvesting stress and consequent metabolic activity necessary for latex regeneration after tapping (Chrestin et al. 1984). When ROS-scavenging systems cannot offset ROS accumulation, cellular dysfunctions lead to the agglutination of rubber particles (Putranto et al. 2015b; Wititsuwannakul et al. 2008a). This physiological syndrome, called Tapping Panel Dryness (TPD), is responsible for major losses in natural rubber production (Okoma et al. 2011b) }.

Besides the evidence of ROS involvement in TPD at biochemical level (Zhang et al. 2016b), several recent transcriptomic analyses reported that the expression of genes involved in the production and scavenging of ROS is regulated in latex. For instance, a comparison of two contrasting clones for latex yield showed that antioxidant-related genes are crucial in the regulation of latex regeneration and the duration of latex flow (Chao et al. 2015a). Juvenility was also found to be related to latex production. Latex from self-rooted juvenile clones created by somatic embryogenesis showed more differentially expressed genes (DEGs) related to the ROS-scavenging metabolism (Li et al. 2016a). Transcriptomic analysis of a set of rubber clones showed that three and six overexpressed DEGs were involved in ROS production and ROS-scavenging, respectively (Tang et al. 2016). Although all these genes were expressed in latex, several other studies did not report any significant changes in the expression of antioxidant genes in latex (Li et al. 2015; Montoro et al. submitted-b; Wei et al. 2015a). Posttranscriptional regulation by microRNAs was observed for some redox-related genes. Sixty-eight families of miRNAs, conserved between species, were identified in *Hevea*, including 15 with their precursors, and 16 species-specific miRNAs (Gébelin et al. 2012; Gébelin et al. 2013a; Gébelin et al. 2013b; Kanjanawattanawong et al. 2014; Lertpanyasampatha et al. 2012; Lertpanyasampatha et al. 2014). Approximately 1,000 targets were predicted (Gébelin et al. 2012), and only a few targets have been experimentally validated to date (Gébelin et al. 2012; Pramoolkit et al. 2014). All these studies globally analysed gene expression but did not specifically check redox-related gene families.

The available *Hevea* genome and transcriptomic datasets provide an opportunity for a comprehensive analysis of redox-related genes, particularly in latex producing tissues. This paper sets out to identify the most important gene families involved in the production and scavenging of ROS in *Hevea brasiliensis,* based on the new complete reference genome sequence (Tang et al. 2016) on the one hand and, on the other hand, the regulation of gene expression in latex, based on one of the most complete transcriptome analyses for a TPD-susceptible clone (Montoro et al. submitted-b). Of the 161 redox-related genes expressed in latex, 28 genes were shown to be targeted by microRNAs or phased siRNA through small RNA and degradome analyses. A working model was proposed for transcriptional and post-transcriptional regulation with respect to the predicted subcellular localization of deduced

proteins. To our knowledge, this paper reports on the most complete classification of redox-related genes for a crop species, and reveals a larger number of genes than in *Arabidopsis*.

Results

Identification and classification of redox-related genes in *Hevea*

Hevea redox-related genes were identified in the rubber genome sequence from clone Reyan 7-33-97 using *Arabidopsis thaliana* amino acid sequences from 30 gene families downloaded from the UniProt database according to the procedure described in Figure 1. *Hevea* genes were compared to eight other species based on a bibliographical analysis (Table 1). This analysis revealed that the redox-related gene families identified mostly dealt with ROS production and scavenging and partial information is available for antioxidant biosynthesis. The number of genes for each species was extracted from the following references: respiratory burst oxidase homologue (Rboh), catalase (Cat), superoxide dismutase (SOD), ascorbate peroxidase (APx), glutathione peroxidase (GPx), monodehydroascorbate reductase (MDHAR), glutathione reductase (GR), dehydroascorbate reductase (DHAR), and peroxiredoxin (Prx) for all species (Mittler et al. 2011), glutathione reductases from *Arabidopsis thaliana* and *Oryza sativa* (Trivedi et al. 2013), peroxidases from *Arabidopsis* (Tognolli et al. 2002), *Oryza* (Passardi et al. 2004), *Populus trichocarpa* and *Sorghum bicolor* (Mathé et al. 2010), methionine sulfoxide reductases (MSR) from *Arabidopsis*, *Populus* and *Oryza* (Rouhier et al. 2006) and *Zea mays* (Zhu et al. 2015), NADPH-dependent thioredoxin reductases (NTR) from *Arabidopsis thaliana*, *Populus*, *Vitis vinifera*, *Oryza* and *Sorghum* (Chibani et al. 2009), glutathione S-transferase (GST) from *Arabidopsis* (Dixon and Edwards 2010), *Populus* (Lan et al. 2009), *Zea* (Chi et al. 2011), *Oryza* (Chi et al. 2011) and *Sorghum* (Chi et al. 2011), glutaredoxin (Grx) from *Arabidopsis* (Belin et al. 2015), *Populus (Couturier et al. 2009)*, *Vitis (Couturier et al. 2009)*, *Oryza (Couturier et al. 2009)* and *Sorghum (Couturier et al. 2009)*,thioredoxin (Trx) from *Arabidopsis* (Belin et al. 2015), *Populus*, *Vitis* and *Sorghum* (Chibani et al. 2009), *Zea* and *Oryza* (Nuruzzaman et al. 2012), ascorbate biosynthesis genes (GDP-L-galactose phosphorylase, GDP- mannose pyrophosphorylase, GDP-mannose-3', 5'epimerase, L-galactono-1,4-lactone dehydrogenase, Inositol phosphate phosphatase, L-galactose dehydrogenase, L-gulonolactone oxidase and Myo-inositol oxygenase) from *Arabidopsis* (Ishikawa and Shigeoka 2008), *Vitis* (Cruz-Rus et al. 2010), *Zea* (Sanahuja et al. 2013), *Populus* (Dumont et al. 2014) and *Oryza* (Zhang et al. 2015a), L-galactono-1,4-lactone dehydrogenase from *Oryza* (Yu et al. 2017) and Myo-inositol oxygenase from *Oryza* (Holler et al. 2015), glutathione biosynthesis genes from *Arabidopsis* (Wachter et al. 2005)*, Populus* (Gomez et al. 2004), *Zea* (Choe et al. 2013) and *Oryza* (Dumont et al. 2014), tocotrienol biosynthesis genes from *Arabidopsis* (Hussain et al. 2013) and *Oryza* (Matsuzuka et al. 2013).

Hevea has a much larger number of redox-related genes (407) compared to *Arabidopsis* (306). This is mainly explained by the absence of genes encoding polyphenol oxidase in *Arabidopsis* when *Hevea* genome harboured 6 genes, and by a smaller number of genes encoding glutaredoxin (43), glutathione S-transferase (51) and peroxidase (73) in *Arabidopsis* compared to *Hevea* (51, 77 and 114, respectively). A phylogenetic analysis was carried for gene families involved in ROS production and scavenging (Supplemental Figures 1-17). This analysis revealed several gene duplications for Grx, GST and Px gene families (Supplemental Figures 7, 8, 14).

Table 2. Classification of main redox-related gene families in Hevea brasiliensis compared with several other species: Arabidopsis thaliana, Manihot esculenta, Oryza sativa, Populus trichocarpa, Ricinus communis, Sorghum bicolor, Vitis vinifera, Zea mays.

Function	Gene family	*Arabidopsis*	*Hevea*	*Manihot*	*Oryza*	*Populus*	*Ricinus*	*Sorghum*	*Vitis*	*Zea*
ROS production	Respiratory burst oxidase homolog	10	9	11	9	10	9	13	8	18
	Polyphenol oxidase	0	6	1	2	11	1	8	4	6
	Peroxidase	73	114	-	138	87	-	140	-	-
ROS scavenging and regulation	Catalase	3	5	10	3	4	2	2	6	3
	Superoxide dismutase	8	9	16	7	10	8	5	12	11
	Ascorbate peroxidase	7	8	19	11	11	10	8	8	16
	Glutathione peroxidase	8	10	7	6	7	5	6	5	5
	Glutathione reductase	2	3	5	3	3	3	2	2	2
	Monodehydroascorbate reductase	5	6	6	5	3	3	5	3	4
	Dehydroascorbate reductase	4	3	3	2	4	4	2	3	2
	Methionine sulfoxide reductase	14	9	-	7	9	-	-	-	6
	Peroxiredoxin	10	10	9	11	12	7	6	9	6
	NADPH-dependent thioredoxin reductase	3	3	-	3	3	-	3	2	-
	Glutathione S-transferase	51	77	-	84	81	-	99	-	72
	Glutaredoxin	43	51	-	49	38	-	32	25	-
	Thioredoxin	38	54	-	46	45	-	29	32	23
Ascorbate biosynthesis	GDP-L-galactose phosphorylase	2	3	-	1	2	-	-	2	1
	GDP- mannose pyrophosphorylase	3	2	-	3	-	-	-	1	-
	GDP-mannose-3′,5′ epimerase	1	2	-	2	2	-	-	2	-
	L-galactono-1,4-lactone dehydrogenase	1	1	-	2	1	-	-	1	1
	Inositol phosphate phosphatase	1	2	-	1	3	-	-	1	1
	L-galactose dehydrogenase	1	3	-	1	2	-	-	2	1
	L-gulonolactone oxidase	7	3	-	-	-	-	-	1	-
	Myo-inositol oxygenase	5	3	-	1	-	-	-	2	-
Glutathione biosynthesis	Glutamate cysteine ligase	1	2	-	1	2	-	-	-	1
	Glutathione synthetase	1	2	-	-	2	-	-	-	1
	Tocotrienol cyclase	1	1	-	1	-	-	-	-	-

	Tocotrienol γ-methyltransferase	1	2	-	1	-	-	-	-	-
Tocotrienol biosynthesis	MPBQ/MSBQ methyltransferase	1	3	-	1	-	-	-	-	-
	Homogentisate phytyltransferase	1	1	-	2	-	-	-	-	-
In total		306	407	-	403+	-	-	-	-	-

Comparative analysis of published latex transcriptomes

In order to identify redox-related genes expressed in latex, contigs or unigenes annotated as redox-related genes were extracted from the Supplemental Table 1 of recently published latex transcriptome analyses obtained by RNA sequencing technology (Chao et al. 2015a; Li et al. 2015; Li et al. 2016a; Montoro et al. submitted-b; Tang et al. 2016; Wei et al. 2015a). For each publication, redox-related contigs or unigenes were assigned to one of the 30 gene families using their initial blastx annotation (Table 2). A small number of contigs (28, 30 and 12) was counted for three studies (Chao et al. 2015a; Li et al. 2015; Li et al. 2016a) compared to the total gene number found in this work (see Table 1) and other transcriptome analyses (912, 77, 231) (Montoro et al. submitted-b; Tang et al. 2016; Wei et al. 2015a). The transcriptome published by Wei and collaborators had the largest number of redox-related contigs (234) but a lower coverage (0.37 Gb for all samples) (Wei et al. 2015a). This transcriptome was obtained from trees of rubber clone RRIM 600 with long-term latex flow. For several gene families, the number of contigs was larger than the gene number counted in the reference genome. Tang and co-workers published transcriptome data for a mixture of several tissues including latex. Thus, the RNAseq dataset from clone PB 260 (Montoro et al. submitted-b) was adopted for further analysis for the following reasons: high coverage (6 Gb per sample), largest number of redox-related contigs (912), representation of all gene families, good statistical design with the use of 3 biological replicates, and data from a comparison of latex from healthy and TPD-affected trees.

Table 3. Annotation of Hevea latex redox-related genes from published latex transcriptomes.

Reference	Chao 2015	Li 2015	Wei 2015	Li 2016	Tang 2016	Montoro submitted	This study
Topic	Rubber yield	Rubber yield	Latex flow	Rubber yield	Genome	TPD	Redox
Technology	Hiseq2000	Hiseq2000	Hiseq2500	Hiseq2000	Hiseq2000	Hiseq2000	-
Throughout	35Mb	4.82Gb	0.37Gb	16.7Mbp	1.29Gb	6 Gb	-
Clone	CATAS8-79 PR107	RRIM 600 RY 7-20-59	RRIM 600	CATAS7-33-97 HAIKEN 2	Reyan7-33-97	PB 260	PB 260
Tissue	latex	latex	latex	latex	Mixed tissues	Latex	Latex
Gene family	**Number of contigs or unigenes**						
Respiratory burst oxidase homolog	1	1	4	0	1	26	2
Polyphenol oxidase	2	0	1	1	1	4	2
Peroxidase	6	2	18	2	5	145	7
Catalase	0	0	8	1	3	31	3

Superoxide dismutase	2	0	14	1	1	43	6
Ascorbate peroxidase	1	0	15	0	1	27	5
Glutathione peroxidase	0	1	10	0	1	45	7
Glutathione reductase	3	0	4	0	1	17	3
Monodehydroascorbate Reductase	1	1	5	0	2	26	5
Dehydroascorbate reductase	0	2	3	0	1	7	3
Methionine sulfoxide reductase	1	1	7	0	3	24	7
Peroxiredoxin	2	0	12	0	2	42	7
NADPH-dependent thioredoxin reductase	0	2	2	0	7	8	3
Glutathione S-transferase	5	6	44	3	16	93	23
Glutaredoxin	2	2	16	0	4	104	20
Thioredoxin	0	10	43	3	21	189	32
GDP-L-galactose phosphorylase	0	0	4	0	1	3	2
GDP- mannose pyrophosphorylase	0	0	0	0	0	3	2
GDP-mannose-3′,5′ epimerase	0	1	3	0	1	7	2
L-galactono-1,4-lactone dehydrogenase	0	0	3	0	0	7	1
Inositol phosphate phosphatase	0	0	0	0	0	7	2
L-galactose dehydrogenase	0	0	0	0	1	3	3
L-gulonolactone oxidase	0	0	2	0	0	16	1
Myo-inositol oxygenase	1	0	2	1	0	7	3
Glutamate cysteine ligase	1	0	2	0	0	5	2
Glutathione synthetase	0	0	1	0	0	4	2
Tocopherol cyclase	0	0	1	0	0	4	1
Tocopherol γ-methyltransferase	0	0	2	0	0	6	1
MPBQ/MSBQ methyltransferase	0	0	3	0	2	3	3
Homogentisate phytyltransferase	0	1	2	0	2	6	1
Total contigs or unigenes	28	30	231	12	77	912	161

Transcriptional regulation of redox-related genes and prediction of subcellular localization in laticifers

Of the 407 *Hevea* redox-related genes, 161 unique transcripts were found in latex (Supplemental Table 1). All transcripts were encoded by a unique gene, except for 3 transcripts encoded by two genes harboured by 2 different scaffolds, respectively: CL1895Contig4 (L-galactose dehydrogenase 1 (GDH1) and L-galactose dehydrogenase 2 (GDH2)), CL3344Contig2 (glutathione S-transferase U8 (GSTU8) and glutathione S-transferase U11 (GSTU11)) and CL2806Contig1 (NADPH-dependent thioredoxin reductase 1 (NTR1) and NADPH-dependent thioredoxin reductase 3 (NTR3)). NTR1 and NTR3 were located on scaffold0536_346249 and scaffold0965_30248. GSTU8 and GSTU11 were located on scaffold0702_452766 and scaffold0702_454607. GDH1 and GDH2 were located on

scaffold1364_78602 and scaffold1364_29743. The phylogenetic analyses revealed a recent duplication of the genes (Supplemental Figures 4, 8, 11).

Subcellular localization of redox-related genes was performed using WoLF PSORT, CELLO2GO and Plant-mPLoc. The largest number of proteins was predicted in chloroplast. Given that laticifers are non-photosynthetic tissues, chloroplast and plastid predictions were assigned as plastidic proteins. Subcellular localization of latex proteins was predicted as follows: 82 in plastids, 70 in cytosol, 12 in nucleus, 7 in mitochondria, 2 in extracellular, 1 in vacuole, 2 in peroxisome and 7 non-predicted.

When exploring RNAseq data from latex(Montoro et al. submitted-b), sixty transcripts were abundant (>1000 reads), and twelve of them were very abundant (>5000 reads) for one or other of the conditions. Twenty-nine transcripts were induced and forty-eight repressed in response to ethephon in healthy trees. Nine transcripts were induced in response to ethephon in TPD-affected trees. Four of these genes (PPO2, PrxQ, TrxS12 and TrxS13) showed contrasting regulation: repressed in healthy and overexpressed in TPD-affected trees. For the clarity of this manuscript, gene expression data are presented in Figures 2, 3 and 4 and commented on in the discussion.

Small RNA-mediated post-transcriptional regulation of redox-related genes

Redox-related transcripts targeted by microRNAs and phased siRNA were searched using CLEAVELAND pipeline (Piyatrakul et al. 2014b) in the degradome dataset obtained from various tissues (root, leaf, bark, latex, flowers and embryo) and the reference transcriptome for rubber clone PB 260. Of the 407 redox-related genes, 13 were targeted by 12 different microRNAs (Table 3). The degradome analysis revealed post-transcriptional regulation of these transcripts at spatial level (thanks to tissue-specific libraries) and cleaved transcript abundance level classified into degradome categories. The number of microRNA families was different for each tissue: 1 in roots, 7 in leaves, 7 in latex, 4 in bark and 2 in flowers. In latex, seven of these redox-related genes were targeted by 6 families of microRNAs. Three known families of microRNA (miR535, miR398b and miR394) targeted and cleaved transcripts from genes *APX3* (ascorbate peroxidase 3), *SOD2* (Cu/Zn superoxide dismutase 2), *GR1* and *GR2* (glutathione reductase 1 and 2), respectively. For transcripts from gene *APX3*, strong spatial regulation was observed with a greater abundance of miR535 in leaf (degradome category 0) compared to latex (degradome category 4). One transcript, encoding MPBQ/MSBQ methyltransferase 3 involved in tocotrienol biosynthesis, was also cleaved in bark and leaf by a new microRNA named miRNAn7. For miR398b, which cleaves chloroplastic Cu/Zn superoxide dismutase transcripts (Gébelin et al. 2013a), a low abundance of cleaved transcripts was found in latex (degradome category 3) and root (degradome category 4). Interestingly, the three cytosolic isoforms were not detected in the degradome libraries confirming the previous observation made by Gébelin and co-workers (Gébelin et al. 2012). The miR398 binding site was further scanned and sequence variations were observed in the 5' and 3' seed region but also between the very sensitive 10^{th} and 11^{th} nucleotides of the miRNA sequence targeting *HbCuZnSOD1* and at the 12^{th} nucleotide targeting both *HbCuZnSOD3* and *HbCuZnSOD4* (Table 4). Three microRNAs from latex library were new microRNAs not annotated yet in the miRBase database (Table 3). Catalase 1, the unique cytoplasmic *Cat* gene showing the highest Cat expression in latex, was regulated by a new miRNA named miRNAn1. This mRNAn1 also targeted peroxisome catalase 2. Cytosolic glutathione reductase 1 and plastidic glutathione reductase 2 were highly expressed in latex and targeted by miR394.

The degradome analysis also revealed post-transcriptional regulations by phased siRNA (Table 5). Analysis with CLEAVELAND pipeline using both 161 ROS-related genes, 6 tissue-specific transcriptomes and annotated phased-siRNA-producing loci led to identify 15

transcripts targeted by 16 different phased siRNA. These transcripts were specifically targeted by phased si-RNA and not targeted by microRNA. Transcripts were cleaved for genes encoding peroxidase 1 and 4 expressed only in latex, MnSOD in root, MDHAR in leaf, methionine sulfoxide reductase in bark and flower, peroxiredoxin in root and leaf, GST in flower, glutaredoxin in bark, thioredoxin in bark and flower. Some other genes involved in ascorbate biosynthesis were post-transcriptionally regulated in bark and leaf, and transcripts from one gene involved in tocotrienol biosynthesis were targeted in leaf.

The expression of the 28 post-transcriptionally regulated genes was recalculated using the reads covering the cleavage site only in order to check if the level of expression assessed by the number of reads describes the real functionality of mRNA (Supplemental Table 1). The expression of 11 of the 28 targeted transcripts were significantly affected by the new way of calculation. Significant fold changes observed in standard RNA sequencing for ethephon treatment or TPD occurrence disappeared for genes *APX3, CAT1, CAT2, CAT3, GR1, MDHAR1, MDHAR2* and *Prx2E* when using reads covering the cleavage site. For *VTE4*, an effect of TPD occurrence appeared, which was not observed when all reads were used. Finally, some effects of TPD occurrence observed in standard condition was exacerbated for *Px1* (under-expression) and *Px6* (over-expression).

Table 4. Degradome data analysis with CLEAVELAND pipeline using 161 ROS-related genes, 6 tissue-specific transcriptomes and newly annotated miRNA.

Target	Degradome			MicroRNA				
Enzyme	**Contig**	**Library**	**Category**	**miRNA accession**	**miRBase annotation**	**Start position**	**Stop position**	**Cleavage site**
Ascorbate peroxidase 3	CL1Contig1117	leaf	0	Pmature12390	miR535	53	73	64
		latex	4	Pmature12390	miR535	53	73	64
Catalase 1	CL1Contig10534	latex	3	Pyoung21016	miRNAn1, in progress	588	608	599
Catalase 2	CL1Contig1382	latex	3	Pyoung21016	miRNAn1, in progress	421	441	432
		latex	4	Pyoung160064	miRNAn2, in progress	422	442	433
Cu/Zn superoxide dismutase 2	CL1553Contig1	root	4	acc_420	miR398b	630	656	646
		latex	3	acc_420	miR398b	630	656	646
Glutathione peroxidase 5	CL449Contig1	leaf	0	Pmature37668	miRNAn3, in progress	70	90	81
		latex	4	Pmature37668	miRNAn3, in progress	70	90	81
Glutathione reductase 1	CL1Contig15684	bark	4	Pyoung83898	miR394	477	500	488
		leaf	2	Pyoung83898	miR394	477	500	488
		latex	2	Pyoung83898	miR394	477	500	488
Glutathione reductase 2	CL1Contig1556	leaf	2	Pyoung83898	miR394	560	583	571
		latex	2	Pyoung83898	miR394	560	583	571
Methionine sulfoxide reductase A2	CL372Contig4	bark	2	health2164	miRNAn4, in progress	210	231	222
		leaf	3	health2164	miRNAn4, in progress	210	231	222
Monodehydroascorbate reductase 1	CL1Contig7966	bark	2	Pmature18863	miRNAn5, in progress	149	170	161
Monodehydroascorbate reductase 3	CL1250Contig6	bark	2	Pyoung84691	miRNAn6, in progress	1181	1203	1194
MPBQ/MSBQ methyltransferase 3	CL5665Contig1	leaf	4	Pyoung169157	miRNAn7, in progress	951	973	962
		flower	2	Pyoung169157	miRNAn7, in progress	951	973	962
Myo-inositol oxygenase 2	CL234Contig10	flower	2	Pyoung68471	miRNAn8, in progress	401	424	415
Peroxidase 6	CL1Contig8355	leaf	2	Pyoung84691	miRNAn9, in progress	970	990	982

Table 5. Comparison of HbmiR398 (acc_420) cleavage site between cytosolic and chloroplastic CuZnSOD isoforms. Arrow indicated the cleavage site observed experimentally for HbCuZnSOD2 by miR398 (Gebelin et al. 2012) and in the degradome analysis. Sequence variations in cytosolic isoforms sequences compared to HbCuZnSOD2 are in bold and highlighted character.

Gene name	Sub-cellular localization	mfe kcal/mol	Alignment
HbCuZnSOD1	cytosolic	Non functional	miRNA 23 UAGU-C-CCCGCUGGACUCU-UGUGU 1 .::: : .:::::..::.:. :.:.. Target 364 GUCAUGC**A**GG**G**GA**UU**UGGG**C**AA**U**AU**U** 390
HbCuZnSOD2	chloroplastic	-37.3	miRNA 23 UAGU-C-CCCGCUGGACUCU-UGUGU 1 .::: : :::.::::::.:: ::::.: Target 456 GUCAUGCGGGUGACCUGGGAAACAUA 480
HbCuZnSOD3	cytosolic	Non functional	miRNA 23 UAGU-C-CCCGCUGGACUCU-UGUGU 1 .::: : .::.::.:::.:: :.:.. Target 346 GUCAUGC**U**GGUGA**U**CUGGGAAA**U**AU**C** 372
HbCuZnSOD4	cytosolic	Non functional	miRNA 23 UAGU-C-CCCGCUGGACUCU-UGUGU 1 .::: : .::.::.:::.:: :.... Target 284 GUCAUGC**U**GGUGA**U**CUGGGAAA**UG**U**C** 309

Table 6. Degradome data analysis with CLEAVELAND pipeline using 161 ROS-related genes, 6 tissue-specific transcriptomes and annotated phased-siRNA-producing loci.

Target		Degradome		siRNA	Phased siRNA			
Enzyme	**Contig**	**Library**	**Category**	**Accession number**	**siRNA annotation**	**Start position**	**Stop position**	**Cleavage site**
Peroxidase 1	CL1Contig4572	latex	4	health_latex_scaffold0023_1034234	phased siRNA n1	807	830	819
Peroxidase 4	CL1Contig19668	latex	1	TPD_latex_scaffold0522_76802	phased siRNA n2	320	342	331
MnSOD1	CL1Contig2591	root	4	health_latex_scaffold0976_205659	phased siRNA n3	14	37	27
Monodehydroascorbate reductase 2	CL1Contig16376	leaf	4	health_latex_scaffold0691_426349	phased siRNA n4	355	376	366
Methionine sulfoxide reductase A2	CL372Contig4	bark	4	young_trees_scaffold0587_304178	phased siRNA n5	623	643	634
Methionine sulfoxide reductase A3	CL372Contig3	flower	2	TPD_latex_scaffold0126_1572060	phased siRNA n6	1003	1022	1013
Peroxiredoxin 2-Cys 1	CL124Contig1	root	4	TPD_latex_scaffold0603_677311	phased siRNA n7	868	887	878
Peroxiredoxin-2E 1	CL1Contig5964	leaf	2	TPD_latex_scaffold0060_279117	phased siRNA n8	1199	1220	1211
Glutathione S-transferase F1	CL1Contig12879	flower	4	young_trees_scaffold0025_2131961	phased siRNA n9	785	805	796
Glutaredoxin 4CxxC1	CL15621Contig1	bark	4	health_latex_scaffold0493_317933	phased siRNA n10	56	74	66
Thioredoxin ACHT5	CL1Contig21402	bark	4	health_latex_scaffold0201_579530	phased siRNA n11	1353	1372	1363
Thioredoxin F1	CL1Contig16697	flower	3	TPD_latex_scaffold0094_2333306	phased siRNA n12	666	688	679
Thioredoxin M3	CL5346Contig1	bark	4	TPD_latex_scaffold4122_4087	phased siRNA n13	667	686	678
GDP-D-mannose pyrophosphorylase 2	CL1Contig4217	bark	4	health_latex_scaffold0418_885165	phased siRNA n14	479	499	490
GDP-D-mannose pyrophosphorylase 2	CL1Contig4217	leaf	4	health_latex_scaffold0118_410750	phased siRNA n15	479	499	490
Tocopherol gamma-methyltransferase	CL1409Contig4	leaf	4	health_latex_scaffold0166_1071486	phased siRNA n16	363	384	374

Discussion

Based on the RNAseq dataset (Supplemental Table 1), smallRNA/target identification (Tables 3-5), and subcellular localization of redox-related genes (Supplemental Table 1), a model of transcriptional and post-transcriptional regulations of redox-related genes was attempted for each subcellular component of the latex cell for rubber clone PB 260 (Figures 2, 3 and 4).

Regulation of ROS production and scavenging in lutoids

Lutoids are polydispersed vacuoles with lysosomal properties, which are considered as the main source of ROS (d'Auzac and Jacob 1989). The subcellular localization of lutoid proteins could not be predicted using WoLF PSORT, CELLO2GO or Plant-mPLoc, which are set up for the main cellular compartments including cytoplasm, nucleus, mitochondrion, chloroplast, plastid membrane, extra-cellular compartments and vacuole. Consequently, the assignment of deduced proteins to lutoids was carried out based on published biochemical information. NADPH-cytochrome-c-reductase was found on the outer surface of lutoid membrane functioning as NADH-O_2 reductase generating superoxide ions released in cytoplasm (d'Auzac et al. 1982b). Respiratory burst oxidase is the key enzymatic subunit of plant NADPH oxidase (Torres and Dangl 2005). *Rboh1* and *Rboh2* transcripts were predicted to target plastid membranes (Figure 2). *Rboh2* transcripts were much more abundant than *Rboh1* (871 vs 6 reads) and were accumulated in response to ethephon in healthy trees (1467 reads) but not when TPD occurred (914 reads). Previous biochemical studies revealed that NADPH oxidase is the main source of ROS in laticifer cytosol, especially under stress (Chrestin et al. 1984). The present study revealed that this enzyme was mostly encoded by the *Rboh2* gene in latex cells, and its expression was enhanced by ethephon application. These results suggest that *Rboh2* encodes the main enzyme generating ROS at the outer surface of its membrane in contact with cytosol. The activity of ROS-scavenging enzymes (peroxidase and catalase) was also localized in lutoid (Coupé et al. 1972a). Surprisingly, of the seven genes encoding peroxidase expressed in latex, three did not carry a signal peptide that could enable the prediction of subcellular localization (Px3, Px4 and Px7). Only Px5 gene, which was slightly expressed, was predicted in lutoid. Lastly, although none of the three *Cat* genes expressed in latex could be identified as a gene encoding a catalase localized in lutoid, Coupé and co-workers reported that 50% of Cat activity was driven by some kind of particles (probably lutoids) and the rest by cytosol (Coupé et al. 1972b).

Regulation of ROS-scavenging systems in cytoplasm

The superoxide anion and hydrogen peroxide are detoxified in latex cytoplasm through enzymatic and antioxidant pathways (Figure 2). Superoxide dismutase is the enzyme involved in the first step of detoxification inducing the dismutation of the superoxide anions, produced by the lutoid NADPH oxidase, into hydrogen peroxide (Zhang et al. 2016b). There were three genes (*CuZnSOD1*, *CuZnSOD3*, *CuZnSOD4*) predicted to encode a cytosolic Cu/Zn superoxide dismutase (Supplemental Table 1). Over-expression of *HbCuZnSOD4* in *Hevea* transgenic plants increases plant growth and tolerance to water deficit but no effect on laticifers was found in that study (Leclercq et al. 2012). *CuZnSOD1* transcripts were much more abundant compared to the other two (1782, 435, and 530 reads, respectively). The *CuZnSOD1* and *CuZnSOD4* genes were under-expressed by ethephon treatment. For the *CuZnSOD1* gene, transcript abundance was comparable in the control (1782 reads) and TPD-affected ethephon-treated trees (1505 reads). Given that superoxide dismutase activity was reported in latex (d'Auzac and Jacob 1989), these results suggest that CuZnSOD1 might play a major role in the detoxification of superoxide anions in cytosol. Unlike *Arabidopsis*, the three cytosolic isoforms are not targeted by miR398 (Gébelin et al. 2012). This absence of post-transcriptional regulation by miR398 is probably due to a mutation in or near the cleavage site (Table 4). To our knowledge, this unique feature has never been reported in the literature. This suggests that *Hevea* miR398 lost this function or did not acquire it because of selection pressure related to the high production of anion peroxide produced by the NADPH oxidase in latex.

The second step deals with the decomposition of H_2O_2 to H_2O and O_2 through five hydrogen peroxide scavenging pathways coexisting in cytosol (peroxidase, ascorbate peroxidase, glutathione peroxidase, peroxiredoxin and catalase) (Figure 2). Of the 7 genes

encoding a peroxidase, only *Px1* was predicted to encode a cytosolic protein. The targeting of these transcripts by phased siRNAn1 in latex might explain the very low level of expression of this gene in latex observed in latex whatever the treatment. Consequently, Px1 activity might be considered to have a minor role in ROS detoxification. The decomposition of H_2O_2 can also be brought about by peroxiredoxin. Two genes encoding a cytoplasmic peroxiredoxin were actively transcribed: *Prx2B1* and *Prx2B2* were under and overexpressed in response to ethephon, respectively, and the occurrence of TPD had no significant effect on their expression levels (Supplemental Table 1). Upstream peroxiredoxin, the first steps of the NTR-Trx-Prx pathway involved Trx encoded by a large family. The *TrxH4*, *TrxH5, TrxH6*, *Nrx2* and *Nrx4* genes were highly expressed under all conditions, and four genes (*TrxH2, TrxH3, TrxH4* and *Nrx4*) were overexpressed by ethephon stimulation. For the genes encoding thioredoxins, *TrxH5* had the highest level of expression (30280 reads) out of the 161 genes expressed in latex (Supplemental Table 1). From our knowledge, there is no published information related to the potential role of Prx in latex and further characterization is required.

By contrast, the ascorbate/glutathione cycle involving in its last lines APx and GPx is essential in the reduction of H_2O_2 to H_2O and O_2 (Figure 2). The transfer of electrons from NADPH to the APx and GPx enzymes requires the reduction power of the reduced antioxidants ascorbic acid (ASA) and glutathione (GSH). Regeneration of the ASA and GSH forms reduced by the ascorbate-glutathione cycle involved several enzymes encoded by *MDHAR2*, *DHAR2*, *GR1* and *GR2*. The ethephon treatment did not transcriptionally activate genes involved in the glutathione/ascorbate cycle. However, some post-transcriptional regulations appeared in the degradome analysis showing that both the *GR1* and *GR2* transcripts were cleaved by miR394 (Table 3). In *Arabidopsis*, mir394 silences LEAF CURLING RESPONSIVENESS, which encodes an F-box protein (SKP1-Cullin/CDC53-F-box), and is essential for maintaining adaptive response to salt and drought stress (Song et al. 2013). APx has a high affinity for H_2O_2 and can reduce it to H_2O in chloroplasts, cytosol, mitochondria and peroxisomes, as well as in the apoplastic space, utilizing ascorbate as a specific electron donor. Of the three genes encoding a cytoplasmic ascorbate peroxidase, the *HbAPx1* and *HbAPx5* transcripts were the most abundant, and only *APx1* was under-expressed in response to ethephon. Considering the lower expression of these 3 *APx* genes compared to the plastidic *APx4* (Supplemental Table 1), the cytosolic ASA pathway might have a lower reducing capacity than the plastid pathway, which is obvious since the production of ROS is known to be high in plastids. *HbGPx3* was the main expressed gene encoding glutathione peroxidase, which was not regulated by ethephon in healthy or TPD-affected trees. Numerous genes encoding a cytosolic glutathione S-transferase were identified. GST is known to catalyse the conjugation of GSH to xenobiotic molecules as well as compounds of endogenous origin (Licciardello et al. 2014; Lan et al. 2013; Rezaei et al. 2013). Of the 23 *Hevea* genes encoding a GST, 21 were predicted as cytosolic GST (Supplemental Table 1). Among them, the *GSTU3* and *GSTF1* genes were actively expressed in latex cells whatever the rubber tree growing conditions, and *GSTF2* was induced by ethephon in healthy and TPD-affected trees. Although transcripts of *GSTF1* gene were targeted by phased siRNAn9, no change in expression was noticed in response to ethephon and TPD occurrence (Supplemental Table 1). A membrane binding *MGST* gene was induced by ethephon in heathy trees (Supplemental Table 1). As GST plays a central role in the use of the reduction power of GSH to detoxify electrophiles, glutathione might be considered as the most important antioxidant in laticifers. GSH and cysteine are the main thiols in latex. The thiol concentration decreases in TPD-affected trees (Putranto et al. 2015b), and it is used as a parameter of latex diagnosis to predict the risk of TPD occurrence in rubber plantations (Eschbach et al. 1984).

Because of their much higher affinity for hydrogen peroxide (Halliwell 1974), APx, GPx and Prx can scavenge hydrogen peroxide more efficiently than catalase at low hydrogen

peroxide concentrations. For that reason, catalase is considered to take over detoxification in the case of high ROS production which cannot be overcome by other ROS-scavenging systems. However, a high level of Cat activity was identified in latex cytosol for a long time (de Haan-Homans 1950b). Of the three *Cat* genes, only *Cat1* encoded a cytosolic catalase. Being expressed at a high level and over-expressed in response to ethephon, *Cat1* transcripts were also shown to be targeted by a new microRNA, named miRNAn1, revealing post-transcriptional regulation of this gene. High and steady ROS production in latex cells requires Cat activity, which generally comes into play under stress. However, low Cat activity was recorded in TPD-affected trees (Chrestin 1989c), enabling the general oxidative stress in latex cells.

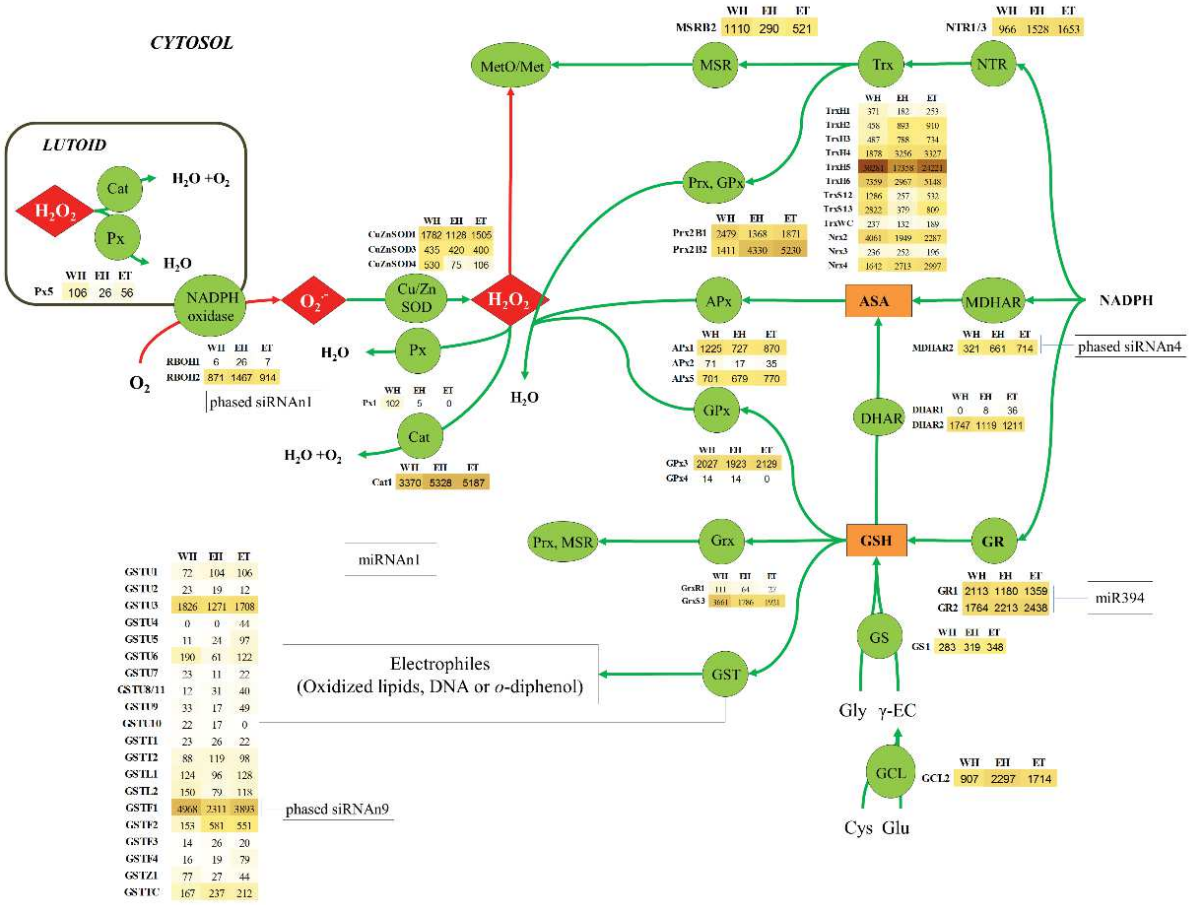

Figure 2. ROS production, scavenging and regulation in lutoid and cytoplasm and glutathione biosynthesis in latex cells. The red arrows represent ROS production or oxidation events. The green arrows represent ROS scavenging reactions or reduction events. Abbreviations are: superoxide radicals ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), catalase (Cat), peroxidase (Px), ascorbate peroxidase (APx), glutathione peroxidase (GPx), peroxiredoxin (Prx), glutathione (GSH), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), glutathione reductase (GR), glutamate cysteine ligase (GCL), glutathione synthetase (GS), γ-glutamylcysteine (γ-EC) , L-glutamate (Glu), cysteine (Cys), glycine (Gly), NADPH reductase (NTR), thioredoxin (Trx), methionine sulfoxide (MetO), methionine sulfoxide reductase (MSR), glutaredoxin (Grx), glutathione S-transferase (GST). The gene expression level is represented using RNAseq reads. The data in the three columns originate from heathy trees without ethephon treatment (WH), heathy trees with ethephon treatment (EH) and tapping panel dryness trees with ethephon treatment (ET) respectively. The CAT1 gene is targeted by miRNAn1. The GR1 and GR2 genes are both targeted by miR394.

Regulation of antioxidant biosynthesis

Glutathione, ascorbate and vitamin E isomers are the major antioxidants in latex (Zhang et al. 2016b). ASA and thiols were found at similar high levels in latex cytosol at 2.9±1.0 mM and 2.2 mM±0.5 mM, respectively (d'Auzac and Jacob 1989). In fact, thiol contents depend on the rubber clone and harvesting conditions, and can be found at a much lower concentration (Putranto et al. 2015b). In this study, a gene expression analysis and prediction of subcellular localization led to a better understanding of the regulation of antioxidant biosynthesis pathways in latex. Glutathione is a tripeptide of glutamic acid, cysteine and glycine (Sies 1999). The glutathione biosynthesis pathway involves two ATP-dependent enzymes: γ–glutamate cysteine ligase (GCL) and glutathione synthetase (GS). GCL combines glutamate and cysteine to give a dipeptide, and then a glycine residue is added to the C-terminal to form glutathione. The limiting factors in this reaction are cysteine concentration and GCL enzyme activity (Noctor et al. 2011). Of the two GS and GCL genes identified in the rubber genome (Table 1), only one of each was expressed in latex cytosol (GS1 and GCL2; Supplemental Table 1 and Figure 2), one GS (GS2) and the two GCL (GCL1 and GCL2) were expressed in plastids. The genes encoding GS2 and GCL2 were significantly over-expressed in response to ethephon. The localizations of these GSs and GCLs suggest that glutathione biosynthesis takes place both in cytosol and plastid as other higher plants (Hell and Bergmann 1990) and in particular *Arabidopsis* (Noctor et al. 2012).

Although ascorbate is present in all plant tissues and cell compartments (Zechmann et al. 2011), it is known to be synthesized in mitochondria (Foyer 2015). There are four routes for ASA biosynthesis: the L-galactose pathway, the *myo*-inositol oxygenase pathway, the salvage pathway via L-galactonate, and the L-gulose-pathway. Of these four routes, L-galactose is the major pathway in many plants (Bulley and Laing 2016; Conklin et al. 1999). The L-galactono-1 4-lactone (L-GalL) biosynthesis pathway occurs in cytosol, which consists of five enzymes (VTC1, GME, VTC2, VTC4 and GDH). All genes encoding these enzymes have homologues expressed in latex cytosol (Supplemental Table 1 and Figure 3). Each of these enzymes was encoded by one or two genes at a high (VTC11, GME2, VTC21, VTC22) or medium expression level (VTC12, GDH1, GDH2), except VTC4 which might be a limiting factor. *VTC12* transcripts were targeted by phased siRNAn14 and siRNAn15. The first step of the pathway can usually be catalysed by either VTC1 or VTC2. Two VTC2 proteins were upregulated during the first 5 tappings of re-opened rubber trees (Yujie 2011). A GDP-D-mannose-3′, 5′-epimerase gene and a VTC2-like gene were expressed at higher levels in a super high productive tree (Tang et al. 2013b). The last enzymatic step of ascorbate biosynthesis is the conversion of L-galactono-1, 4-lactone to L-ascorbate by L-galactono-1, 4-lactone dehydrogenase (GLDH). Usually, the reaction takes place on the inner mitochondrial membrane (Pick and Weber 2014). Surprisingly, the unique *Hevea* gene encoding a GLDH was annotated as chloroplastic protein. For this gene, the three tested programs predicting protein subcellular localization provided contradictory results: chloroplast for WoLF PSORT, chloroplast and mitochondria for CELLO2GO, and vacuole for Plant-mPLoc (Supplemental Table 1). In view of the importance of GLDH and the novelty of its subcellular localization in plastids instead of mitochondria, further evidence for subcellular localization of GLDH will be required. The *myo*-inositol oxygenase pathway is considered as an alternative ascorbate biosynthetic pathway in plants. Although potentially functional in *Hevea*, a limiting factor was identified in this pathway, which might play only a secondary role. In *Hevea,* the *myo*-inositol oxygenase pathway consists of genes encoding a myo-inositol oxygenase (*Miox1*, *Miox2*, *Miox3*) and a l-gulonolactone oxidase (*GULO*). All these genes were activated in latex at medium and low levels depending on the treatments, with 78-1389 reads for *Miox*, and 204-393 reads for *GULO* (Figure 3). *Miox2* transcripts were shown to be targeted by phased siRNAn8. In photosynthetic eukaryote

lineages, GULO was functionally replaced with GLDH after plastid acquisition (Wheeler et al. 2015). The low level of expression of the *Hevea GULO* gene suggests a poor capacity for ascorbate production through the *myo*-inositol oxygenase pathway. This transcriptional regulation was identified as a major regulation way since ascorbate levels could be increased 2- to 3-fold in *miox4*-over-expressing *Arabidopsis* lines (Lorence et al. 2004). Transcriptional regulation of the *Hevea GULO* gene might occur through a weak promotor. However, little is known about the regulation of ascorbate biosynthesis (Bulley and Laing 2016). Lastly, no post-transcriptional control was identified by microRNA for the *Hevea GULO* gene.

There are 4 vitamin E isomers in latex: α-tocopherol, α-tocotrienol, γ-tocotrienol and δ-tocotrienol (Dunphy et al. 1965; Yacob et al. 2012). Genes involved in the biosynthesis of δ-tocotrienol (*VTE1*and *VTE2*) and γ-tocotrienol (*VTE1, VTE31*, *VTE32* and VTE33) were expressed at moderate or high levels in latex (Figure 3). By contrast, *VTE4* involved in the conversion of γ-tocotrienol into α-tocotrienol was expressed at a low level because it is targeted by phased siRNAn16, suggesting that *VTE4* might be a limiting factor. VTE33 had also a low level of expression related to its targeting by miRNAn7. Although VTE5 plays an important role in the *Arabidopsis* tocopherol biosynthesis pathway (Valentin et al. 2006), transcripts encoding phytol kinase (VTE5) were not detected in latex. As γ-tocotrienol is the most abundant vitamin E isomer, its accumulation could be fostered by the weak capacity to produce α-tocotrienol and tocopherol.

Regulation of ROS-scavenging systems in plastids

A laticifer is a non-photosynthetic tissue containing plastids instead of real chloroplasts. In this discussion, the subcellular localization of proteins predicted in chloroplast was therefore assigned as laticifer plastids (Supplemental Table 1). Frey-Wyssling particles are the main plastidic component in the laticifer of mature trees. These specialized chromoplasts have a large amount of carotenoids and lipids (de Faÿ et al. 1989), and *o*-diphenol oxidase activity (Coupé et al. 1972b). *o*-diphenol oxidase is a specific polyphenol oxidase (PPO), which is the second source of ROS in latex (Coupé et al. 1972b). PPO utilizes molecular oxygen to catalyse the hydroxylation and dehydrogenation of phenolic compounds to form reactive *o*-quinones. PPO is encoded in latex by two genes, *PPO1* and *PPO2,* with *PPO1* transcripts being more abundant than *PPO2* transcripts. Chloroplastic subcellular prediction led to these genes being assigned to a plastidic compartment (Figure 3; Supplemental Table 1). Surprisingly, Wititsuwannakul and co-workers argued that PPO activity was found at a much higher level in lutoids than in Frey-Wyssling particles (Wititsuwannakul et al. 2002). This report was based on the fact that bottom fraction of latex serum only contains lutoids. However, Coupé and co-workers reported that o-diphenol oxidase could not be located in lutoids because its activation curve is different from that of phosphatase acid. In addition, isopycnic centrifugation revealed that PPO was related to Frey-Wyssling particles (Coupé et al. 1972b); the latter information confirms our sequence analysis.

Peroxidation of Frey-Wyssling particle membranes can lead to the release of oxidases involved in the coagulation of latex (Bzrozowska et al. 1974; Hanover et al. 1976). The first stage of the ROS-scavenging system concerns the dismutation of superoxide anions into hydrogen peroxide by SOD (Figure 3). *CuZnSOD2* was the unique gene encoding a plastidic Cu/Zn superoxide dismutase 2. The weak expression of this gene was related to the targeting of these transcripts by miR398b leading to mRNA cleavage (Gébelin et al. 2013a). The absence of major production of superoxide anions in latex plastids is related to the low SOD detected activity. In contrast, the detoxification of H_2O_2 was supported by the presence of several enzymes, including Prx, APx and GPx.

Peroxiredoxins (Prx) are ubiquitous thiol-dependent peroxidases interacting with a large number of proteins (Cerveau et al. 2016). Prx, also termed thioredoxin peroxidases and alkyl-hydroperoxide-reductase-C22 proteins, are known to participate in the maintenance of low steady-state levels of H_2O_2. Prxs use redox-active cysteines to reduce peroxides and were originally divided into three classes based on the number of cysteinyl residues directly involved in catalysis: 1-Cys, 2-Cys and atypical 2-Cys (Wood et al. 2003). Enzymes involved in this detoxification pathway were found to be encoded by a large family of Trx (19 genes), and some small gene families *MSR* (4 genes encoding a methionine sulfoxide reductase), *NTR* (1 gene), and *Prx* (3 genes). Three *Trx* genes (*TrxA5*, *TrxF1* and *TrxM3*) and 2 *Prx* genes (*Prx2C1*, *Prx2E*) were repressed by 5 phased siRNAs. Several members of each family were highly transcribed in latex (*MSRA2, MSRB1, P*rx*2C2, P*rx*2E, T*rx*A4, T*rx*A5, T*rx*O, T*rx*Y2,* etc.*)* except the unique *NTR2* gene encoding a plastidic NTR. For transcripts targeted by phased siRNA, the analysis of the number of reads covering the cleavage site revealed that most of transcripts were cleaved and the large number of reads did not reflect the real level of expression (Supplemental Table 1). However, a plastidic Grx encodes by another large family of 16 genes might be able to reduce Trx and Prx when NTR is absent (Reichheld et al. 2007).

Ascorbate peroxidase and glutathione peroxidase might be crucial components for active ROS detoxification. Several genes of the glutathione-ascorbate cycle (*APx4, GPx3, GPx5* and *GPx7*) encoding plastidic proteins were highly expressed (more than 1,000 reads) (Figure 3). Conversely, the *APx3* gene was expressed at a very low level and cleaved by miR535, which is highly regulated by stress, revealing a post-transcriptional regulation of this gene. The reduction of MDHA into ASA by MDHAR might not be taking place in plastids since all *MDHAR* genes encoding a plastidic MDHAR were poorly expressed.

Glutathione has a pivotal role in ROS detoxification pathways through glutathione peroxidases (GPx), dehydroascorbate reductase (DHAR), glutathione S-transferases (GSTs) and glutaredoxin (Grx). These proteins were encoded by three highly expressed *GPx* genes (*GPx3, GPx5, GPx7*), *DHAR3*, three poorly expressed *GST* genes (*GSTU5, GSTU6, GSTL3*) and 16 *GRX* genes, of which four were highly expressed (*G*rx*R4, G*rx*C2, G*rx*S1* and *G*rx*S3*). GPx5 was targeted by miRNAn3. Glutathione reducing power is regenerated by plastidic GR, which is encoded by three genes, of which two are highly expressed (*GR1* and *GR2*). MDHAR1 and MDHAR3 were targeted by miRNAn5 and miRNAn6 by prediction respectively.

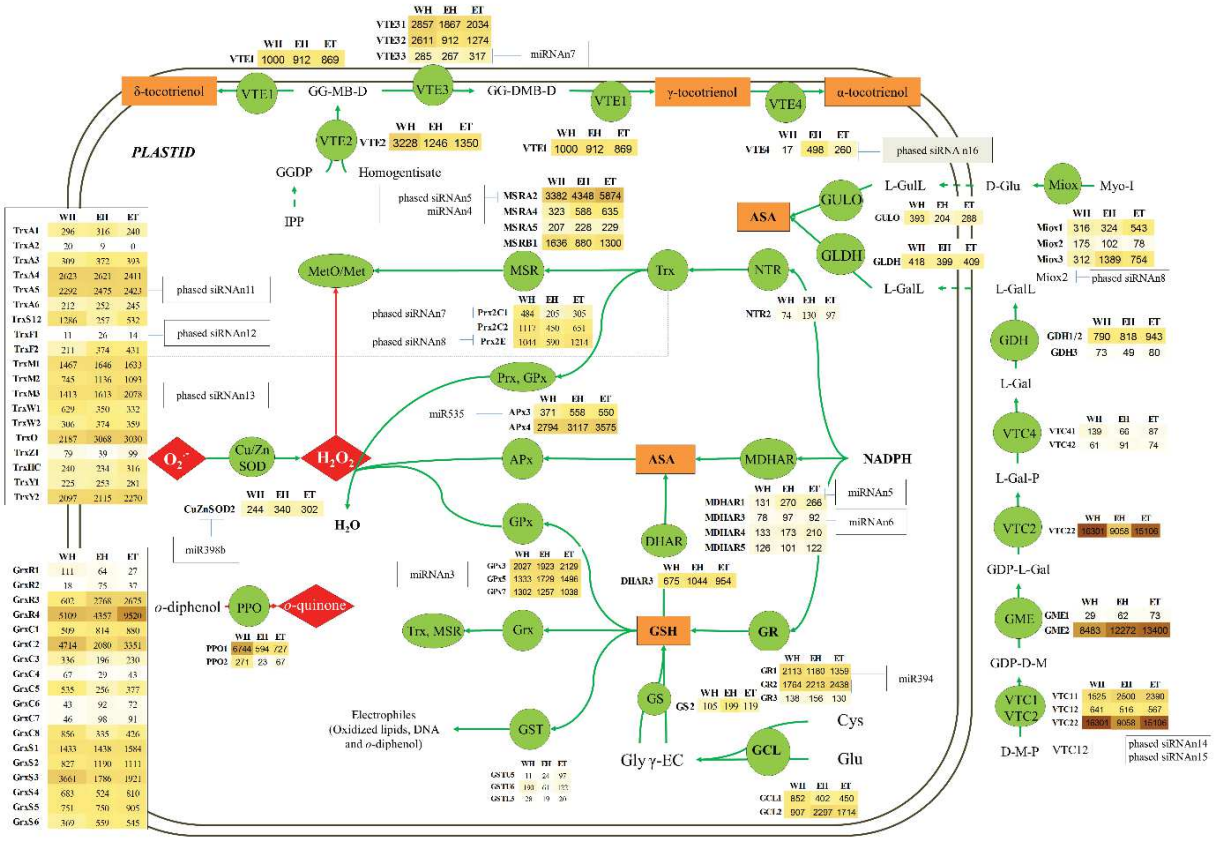

Figure 3. ROS production, scavenging and regulation in plastids and antioxidant biosynthesis in latex cells. The red arrows represent ROS production or oxidation. The green arrows represent ROS scavenging reactions or reduction. Abbreviations are: ascorbate peroxidase (APx), glutathione peroxidase (GPx), peroxiredoxin (Prx), , glutathione (GSH), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), , glutathione reductase (GR), NADPH reductase (NTR), thioredoxin (Trx), methionine sulfoxide (MetO), methionine sulfoxide reductase (MSR), glutaredoxin (Grx), glutathione S-transferase (GST), myo-inositol oxygenase (Miox), L-gulonolactone oxidase (GULO), myo-Inositol (Myo-I), D-Glucuronate (D-Glu), L-Gulono-1, 4-lactone (L-GulL), GDP-D-mannose pyrophosphorylase (VTC1), GDP-L-galactose phosphorylase (VTC2), D-Mannose-1-P (D-M-P), GDP-D-Mannose (GDP-D-M), GDP-mannose 3, 5-epimerase 1 (GME), GDP-D-M, GDP-L-Galactose (GDP-L-Gal), L-Galactose-1-P (L-Gal-P), inositol phosphate phosphatase (VTC4), L-Galactose (L-Gal), L-galactose dehydrogenase (GDH), L-Galactono-1, 4-lactone (L-GalL), L-galactono-1, 4-lactone dehydrogenase (GLDH) , isopentenyl diphosphate (IPP), geranylgeranyl diphosphate (GGDP), homogentisate phytyltransferase (VTE2), 6-Geranylgeranyl-2-methylbenzene-1,4-diol (GG-MB-D), MPBQ/MSBQ methyltransferase (VTE3), 6-Geranylgeranyl-2, 3-dimethylbenzene-1, 4-diol (GG-DMB-D), tocopherol cyclase (VTE1), tocopherol γ-methyltransferase (VTE4), glutamate cysteine ligase (GCL). The gene expression level is represented using RNAseq reads. The data in the three columns originate from heathy trees without ethephon treatment (WH), healthy trees with ethephon treatment (EH) and tapping panel dryness trees with ethephon treatment (ET), respectively.

Regulation of ROS production and scavenging systems in peroxisome, nucleus, mitochondrion and extracellular fluid

Several findings supported the hypothesis that the GSH pathway is the main ROS-scavenging pathway for reducing hydrogen peroxide in mitochondria. Seven genes (*MnSOD1, MnSOD2, APx3, GPx6, GPx7, P*rx*2F* and *NTR2*) encoded ROS-scavenging enzymes localized in mitochondria (Figure 4). The *MnSOD1* and *MnSOD2* genes were highly expressed, but *MnSOD1* transcripts were targeted by phased siRNAn3 and the number of reads mostly consisted of cleaved reads (Supplemental Table 1). These results suggest that only *MnSOD2* is responsible for the SOD production and consequent detoxification of the peroxide anion in mitochondria. There was no or little expression of genes encoding mitochondrial DHAR, MDHAR or thioredoxin, revealing the lack of regeneration for the ascorbate reducing form and peroxiredoxin activity. Although ASA production generally occurs in mitochondria, this pathway is assumed to have an insignificant impact in the mitochondria of latex cells. In addition to the absence of the predicted mitochondrial GLDH involved in the last step of ASA biosynthesis, the low expression of the unique gene encoding an APx (*APx3*) was concomitant with the cleavage of its mRNAs by miR535. In contrast, GPx was encoded by two mitochondrial genes highly expressed in latex (*GPx6*, *GPx7*). *GPx6* was the most highly expressed gene encoding a glutathione peroxidase in latex (Supplemental Table 1). Expression of *MnSOD1* and *GPX6* decreased in response to ethephon but their transcripts remained very abundant. Given the low expression level of the *NTR2* gene and the absence of Trx transcripts, the peroxiredoxin pathway appears not to be essential in the mitochondria of latex cells, as was shown in *Arabidopsis* (Reichheld et al. 2007) .

Genes encoding nuclear peroxidase (*Px6*) and peroxiredoxin (*PrxQ*) being poorly expressed, the glutathione pathway involving a thioredoxin and a methionine sulfoxide reductase might play a major in protein reduction in nucleus (Figure 4). However, upstream this reduction pathway, the two genes *Grx4C1* and *Grx4C2* encoding two nuclear glutaredoxin were weakly expressed and in addition *Grx4C1* transcripts were targeted by phased siRNAn10. Grx activity might be considered as a limiting factor. The two genes encoding a nuclear methionine sulfoxide reductase (*MSRA2* and *MSRA3*) were highly expressed but targeted by miRNAn4, phased siRNAn5 and siRNAn6, respectively. The analysis of read covering cleavage sites revealed that only MSRA2 maintained a high number of reads not cleaved and potentially effective transcripts (Supplemental Table 1). MSR can work with thioredoxin to reduce the oxidative methionine of proteins.

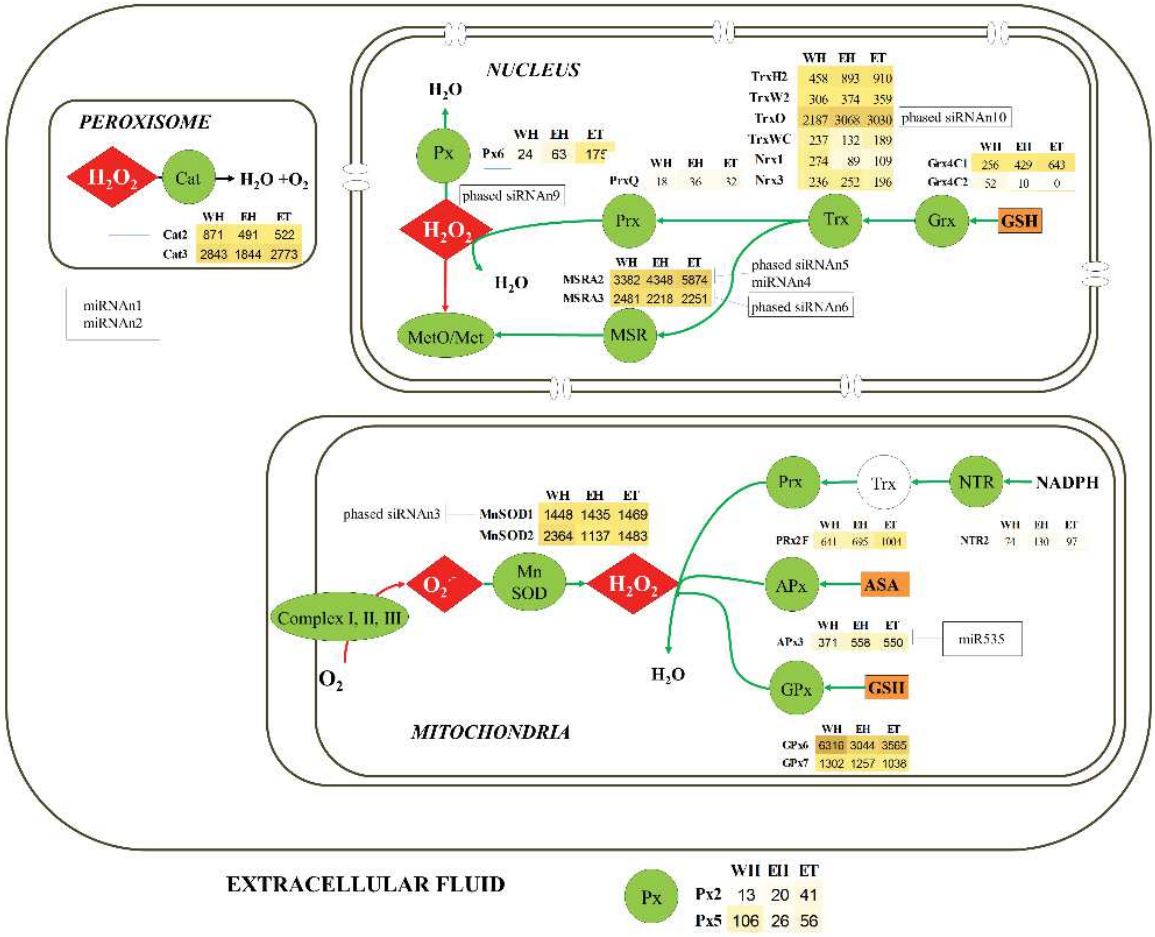

Figure 4. ROS production, scavenging and regulation in the other components of latex cells. The red arrows represent ROS production or oxidation events. The green arrows represent ROS scavenging reactions or reduction events. Abbreviations are: hydrogen peroxide (H_2O_2), catalase (Cat), peroxiredoxin (Prx), peroxidase (Px), glutaredoxin (Grx), thioredoxin (Trx)methionine sulfoxide reductase (MSR), methionine sulfoxide (MetO), superoxide radicals ($O_2^{\bullet-}$), ascorbate peroxidase (APx), glutathione peroxidase (GPx), ascorbate (ASA), glutathione (GSH), NADPH reductase (NTR). The gene expression level is represented using RNAseq reads. The data in the three columns originate from heathy trees without ethephon treatment (WH), heathy trees with ethephon treatment (EH) and tapping panel dryness trees with ethephon treatment (ET), respectively.

Of the 161 *Hevea* redox-related genes expressed in latex, only two genes (*Cat2* and *Cat3*) encoding a catalase were predicted in peroxisome. Expression of *Cat3* was greater than *Cat2*. *Cat3* was under-expressed in response to ethephon treatment in healthy trees but not in TPD-affected trees. Lower expression of *Cat2* might be explained by its post-transcriptional regulation by two microRNAs (Pyoung21016 and Pyoung160064) (Supplemental Table 1). Long fatty acid chains are oxidized in peroxisomes through β-oxidation, which generates acetyl-CoA and hydrogen peroxide. Catalase activity enables the detoxification of that ROS. β-oxidation utilizes substrates imported via ATP-binding cassette (ABC) transporters of subfamily D (Baker et al. 2015; Mhamdi et al. 2012). Two latex ABCD transporters were identified in *Hevea,* and their expression is mediated by jasmonate in laticifers (Zhiyi et al. 2015). These results suggest a functional activity of peroxisome in latex cells. Lastly, the two

genes (*Px2* and *Px5*) encoding a secreted peroxidase into the extracellular fluid were poorly expressed.

Conclusion

Apart from plant model species, this study is the most complete genome-wide analysis of ROS production and scavenging systems and antioxidant biosynthesis in a perennial crop. *Hevea* has 407 redox-related genes, which is a larger number of genes compared to *Arabidopsis*. Transcriptomic, degradome and sequence analyses led to the prediction of their subcellular activities and post-transcriptional regulations through microRNAs. This comprehensive analysis of redox-related genes highlighted critical steps of redox homeostasis in latex cells and led to a list of priority studies being determined for functional analyses. This non-photosynthetic tissue appears to be an interesting model for observing redox homeostasis regulation in response to environmental constraints and in the absence of light stress.

This study also revealed specific regulation of ROS-scavenging systems, which might be adapted to strong and steady ROS production in latex cells due to recurrent harvesting stress and latex regeneration between two tappings. Rboh2 was identified as the main source of ROS in latex. SOD activity is the first line of defence against anion superoxide. Unlike *Arabidopsis*, none of the *Hevea* cytosolic SOD isoforms was subjected to post-transcriptional regulation by miR398. This data might support the maintenance of SOD activity and a consequent high level of anion superoxide dismutation. To overcome this high ROS production in latex cells, strong CAT activity is necessary, which is also controlled by the microRNA miRn1 newly identified in this study. In addition, glutathione and ascorbate are essential antioxidants in latex. As oxidation and degradation of ascorbate are assumed to be faster under light due to ROS generation (Bulley and Laing 2016), further analyses could lead to a better understanding of the regulation of these antioxidants, particularly in response to harvesting and abiotic stress.

To conclude, several genetic studies have revealed the involvement of antioxidant compounds in complex traits of several species (Mellidou et al. 2012; Stevens et al. 2008; Sauvage et al. 2014; Jo and Hyun 2011). In *Hevea*, the 161 redox-related genes expressed in latex represent candidate genes for the identification of allelic variability. The development of molecular markers and the analysis of genetic variability of antioxidants should support breeding programmes, especially for traits relative to environmental stress.

Materials and methods

Identification and classification of redox-related genes in the *Hevea brasiliensis* genome and transcriptome

Redox-related genes were identified from both the *Hevea* reference genome and transcriptome (Figure 1). An amino acid sequence dataset was created by downloading sequences of thirty redox-related gene families from the UniProt database (http://www.uniprot.org/) using published accession numbers mostly from *Arabidopsis,* except for the polyphenol oxidase family, which is absent in *Arabidopsis*. Sixteen families were selected for ROS production and scavenging: respiratory burst oxidase homologue, polyphenol oxidase, catalase, superoxide dismutase, ascorbate peroxidase, glutathione peroxidase, monodehydroascorbate reductase, glutathione reductase, dehydroascorbate reductase, peroxiredoxin (Mittler et al. 2011), glutathione reductase (Trivedi et al. 2013), peroxidase (Tognolli et al. 2002), methionine sulfoxide reductase (Rouhier et al. 2006), NADPH-dependent thioredoxin reductase (Chibani et al. 2009), glutaredoxin and thioredoxin (Belin et al. 2015). In addition, protein sequences of

genes involved in the biosynthesis of three major antioxidants in latex (ascorbate (Ishikawa and Shigeoka 2008), glutathione (Wachter et al. 2005), and tocotrienol (Hussain et al. 2013)) were collected . Lastly, the dataset was completed by the polyphenol oxidase family using *Populus trichocarpa* sequences (Tran et al. 2012). This dataset was blasted against the published *Hevea* genome (Tang et al. 2016) and transcriptome (Montoro et al. submitted-b). Redox-related contigs were also identified using blastx and GO annotations of the *Hevea* transcriptome. The two lists of contigs were merged and blasted on the rubber genome to identify unique contigs. Redox-associated genes were classified for each gene family related to ROS production, ROS-scavenging and regulation, and antioxidant biosynthesis (ascorbate, glutathione and tocotrienols).

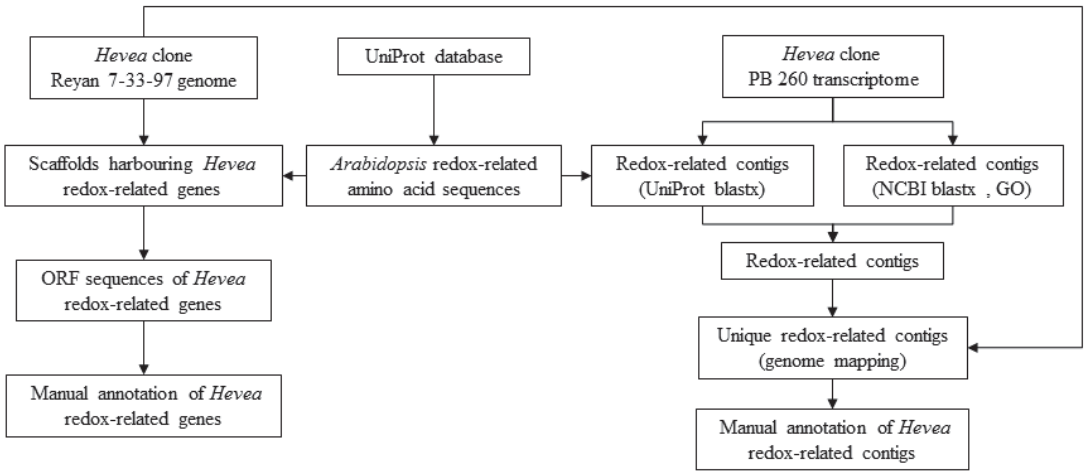

Figure 1. Workflow diagram illustrating the main steps in the identification of redox-related genes in the *Hevea* reference genome sequence and transcriptome. Reference redox-related amino acid sequences were downloaded from the UniProt database. These sequences were blasted against the *Hevea* genome and transcriptome. Scaffolds harbouring *Hevea* redox-related genes were validated manually with ORF. Redox-related contigs were also identified using blastx and GO annotations of the *Hevea* transcriptome. The two lists of contigs were merged and blasted against the *Hevea* genome to identify unique contigs. They were then manually annotated with ORF and genome mapping.

Phylogenetic analysis of redox-related genes

The full length amino acid sequences of *Arabidopsis* redox-related protein were aligned with the amino acid deduced sequences from Hevea clone Reyan 7-33-97 genome. The polyphenol oxidase family being absent in *Arabidopsis*, we used the *Populus* PPO gene family. This alignment was made by Muscle via Mega 6 (Sohpal et al. 2010). Amino acid sequence of Arabidopsis actin 1 or Arabidopsis glutamate cysteine ligase was used as outgroup control. The phylogenetic trees were generated in Mega 6 after alignment.

Prediction of the subcellular localization of redox-related proteins

The subcellular location of redox-related genes was predicted with translated sequences using WoLF PSORT (http://www.genscript.com/wolf-psort.html), CELLO2GO (http://cello.life.nctu.edu.tw/cello2go/) and Plant-mPLoc

(http://www.csbio.sjtu.edu.cn/bioinf/plant-multi/). The 3 predictors were successfully tested on subcellular localization prediction (Xiong et al. 2016). The matching ratio between the prediction result and protein location was calculated according to Xiong's Supplemental Table 1 (Xiong et al. 2016). The matching ratios from these 3 predictors ranged from 50% to 80%. The prediction of subcellular localization was considered as acceptable when the matching ratio of merged results was above 90%.

Identification of small RNA and target mRNA couples

Degradome data for several *Hevea* tissues (latex, leaves, male and female flowers, seeds, root, bark and somatic and zygotic embryos) were obtained according to a protocol adapted from German (German et al. 2009). *Hevea* microRNAs from small RNAseq data published by Gébelin and co-workers (Gébelin et al. 2012; Gébelin et al. 2013a) were annotated by MITP (https://sourceforge.net/projects/mitp/files/). Phased siRNA production sites were predicted by using the UEA small RNA Workbench freeware (Stocks et al. 2012a) (http://srna-workbench.cmp.uea.ac.uk/; software version 4.3.1 Alpha). Degradome data were then analysed using the CLEAVELAND pipeline developed by Addo-Quaye (Addo-Quaye et al. 2009). The degradome categories correspond to : category 4: just one read at this position; Category 3: >1 read, but below or equal to the average depth of coverage on the transcript; Category 2: >1 read, equal to the average depth of coverage on the transcript; Category 1: >1 read, equal to the maximum of the average depth of coverage on the transcript when there is >1 position at maximum value; Category 0: >1 read, equal to the maximum of the average depth of coverage on the transcript when there is just one position at maximum value.

Phased siRNA production sites were predicted using UEA small RNA Workbench freeware (Stocks et al. 2012b) (http://srna-workbench.cmp.uea.ac.uk/; software version 4.3.1 Alpha). Firstly, small RNAs were aligned to the PB260 genome, and non-matching sRNAs were discarded. As input sRNA files, clean reads for latex from healthy trees, TPD-affected trees and young plants were used, with a minimum sRNA abundance of 2, a p-value cutoff of 0.0001 and a length of 21 nt were adopted. The 21-mersphased RNA loci were identified using the TA-SI Prediction tool (p-value cutoff of < 0.0001) of the UEA Small RNA Workbench, and the algorithm of phased siRNA, which calculated the probability of the phasing being significant based on the hypergeometric distribution (Stocks et al. 2012b). At each identified site, coding potential was calculated using CPC software (coding potential calculator, http://cpc.cbi.pku.edu.cn/). Expression of cleaved transcripts related to redox genes were calculated from the same RNA-seq datasets, with the exact number of reads overlapping the sRNA binding site, by using BEDTOOLS program (2.24.0) to intersect bam files with sRNA binding site coordinates (between Tstart and Tstop) provided by CLEAVELAND outputs. Then, by using R package EdgeR, comprising an over-dispersed Poisson model taking into account both biological and technical variability, differential gene expression analyses of replicated count data were performed (Montoro et al. submitted-a). The experimental design allows side-by-side comparison to identify firstly, differentially expressed genes upon ethephon stimulation in the latex of healthy trees, and secondly, differentially expressed genes in the latex of healthy and TPD-affected trees subjected to ethephon stimulation.

Author contributions

YZ classed the gene families, predicted subcellular localization and analysed gene expression data. SW, EOA and SP performed the bioinformatics analyses. JL and SW performed the degradome analysis. CT provided the genome dataset. SH supervised the bioinformatics

analyses. YZ, JL and PM drafted the manuscript. All the co-authors edited and approved the manuscript. PM coordinated the TOSCA project.

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Supplementary Table 1. List of redox-related genes expressed in latex, their location on genomic scaffolds, their predicted subcellular localization, their expression under standard condition (water treatment), ethephon treatment (ET) in healthy or TPD-affected trees (tapping panel dryness)

		Enzyme	ID in genome	ID in transcriptome	Posttranscriptional regulation in latex	Subcellular localization prediction	Corresponding gene	Number of reads covering the total transcript			Fold change		Number of reads covering the cleavage site of transcript			Fold change	
								Water/heathy	ET/heathy	ET/TPD	ET effect	TPD effect	Water/heathy	Ethephon/heathy	Ethephon/TPD	ET effect	TPD effect
ROS production	RBOH1	Respiratory burst oxidase homolog 1	scaffold0702_194727	CL575Contig3		Plastid membrane	AT5G51060	5.75	26.43	7.33	2.20						
	RBOH2	Respiratory burst oxidase homolog 2	scaffold0319_32052	CL7296Contig1		Plastid membrane	AT1G64060	871.42	1467.40	914.32	0.75						
	PPO1	Polyphenol oxidase 1	scaffold1007_118363	CL1Contig10506		Chloroplast	AAU12257.1	6743.72	594.42	727.22	-3.50						
	PPO2	Polyphenol oxidase 2	scaffold1007_214226	CL1Contig4822		Chloroplast	AAU12257.1	270.77	23.12	67.16	-3.55	1.54					
ROS scavenging and regulation	Px1	Peroxidase 1	scaffold0591_141879	CL1Contig4572	phased siRNA n1	Cytoplasm	AT4G21960	101.99	4.51	0.00	-4.50		12.00	1.00	5.00	-3.77	-1.69
	Px2	Peroxidase 2	scaffold0163_231995	CL1Contig11216		Extracellular	AT3G28200	12.81	20.15	40.88							
	Px3	Peroxidase 3	scaffold4235_6773	CL1Contig17826		-	AT1G71695	21.29	14.22	26.96							
	Px4	Peroxidase 4	scaffold0452_857224	CL1Contig19668	phased siRNA n2	-	AT5G40150	209.98	154.13	198.98			31.67	33.00	32.33		
	Px5	Peroxidase 5	scaffold3362_3551	CL1Contig4381		Extracellular,Vacuole	AT1G71695	105.83	25.59	56.24	-2.05						
	Px6	Peroxidase 6	scaffold0471_737858	CL1Contig8355	miRNAn9, in progress	Nucleus	AT5G06720	23.61	62.70	175.15	1.41	1.48	4.33	14.00	47.67		2.93
	Px7	Peroxidase 7	scaffold0246_775027	CL1Contig7348		-	AT2G22420	30.16	15.61	0.00							
	Cat1	Catalase 1	scaffold0073_1899485	CL1Contig10534	miRNAn1, in progress	Cytoplasm	AT4G35090	3369.52	5327.88	5186.72	0.66		403.33	631.00	534.00		
	Cat2	Catalase 2	scaffold0198_214396	CL1Contig1382	miRNAn1, in progress	Peroxisome	AT4G35090	871.34	490.52	521.53	-0.83		397.33	607.33	504.33		
	Cat2	Catalase 2	scaffold0198_214396	CL1Contig1382	miRNAn2, in progress	Peroxisome	AT4G35090	871.34	490.52	521.53	-0.83		388.00	599.67	497.67		
	Cat3	Catalase 3	scaffold0850_174624	CL1Contig6737		Peroxisome	AT4G35090	2842.69	1844.35	2773.49							
	CuZnSOD1	Cu/Zn superoxide dismutase 1	scaffold0407_161864	CL1202Contig5		Cytoplasm	AT5G18100	1781.97	1128.11	1505.19	-0.66						
	CuZnSOD2	Cu/Zn superoxide dismutase 2	scaffold0549_93226	CL1553Contig1	miR398b	Chloroplast	AT2G28190	243.53	340.27	301.76			123.33	162.33	156.67		
	CuZnSOD3	Cu/Zn superoxide dismutase 3	scaffold0718_440390	CL1Contig12224		Cytoplasm	AT1G08830	435.18	419.85	400.49							
	CuZnSOD4	Cu/Zn superoxide dismutase 4	scaffold0117_29717	CL1Contig1628		Cytoplasm	AT1G08830	529.64	74.70	105.73	-2.83						
	MnSOD1	Mn superoxide dismutase 1	scaffold0387_121763	CL1Contig2591	phased siRNA n3	Mitochondrion	AT3G10920	1448.20	1434.80	1469.36			9.33	6.67	6.00		
	MnSOD2	Mn superoxide dismutase 2	scaffold0427_434785	CL1Contig6941		Mitochondrion	AT3G10920	2364.28	1136.52	1482.81	-1.06						
	APx1	Ascorbate peroxidase 1	scaffold4727_3605	CL1Contig8567		Cytoplasm	AT1G07890	1224.53	727.48	869.84	-0.75						
	APx2	Ascorbate peroxidase 2	scaffold0681_172185	CL1Contig9380		Cytoplasm	AT1G07890	71.11	16.91	35.37	-2.07						
	APx3	Ascorbate peroxidase 3	scaffold0041_1336618	CL1Contig1117	miR535	Chloroplast, Mitochondrion	AT1G77490	370.89	558.31	550.24	0.59		41.33	61.67	57.67		
	APx4	Ascorbate peroxidase 4	scaffold3921_6257	CL1Contig4710		Chloroplast	AT4G08390	2793.88	3116.69	3574.84							
	APx5	Ascorbate peroxidase 5	scaffold0050_1276797	CL1063Contig1		Cytoplasm	AT4G35000	701.10	679.10	769.54							
	GPx1	Glutathione Peroxidase 1	scaffold0320_336291	CL908Contig8		-	AT2G48150	115.32	76.63	85.74							
	GPx2	Glutathione Peroxidase 2	scaffold0154_1351240	CL908Contig10		-	AT2G48150	36.03	16.24	29.92							
	GPx3	Glutathione peroxidase 3	scaffold1121_63863	CL355Contig3		Cytoplasm, Chloroplast	AT1G63460	2027.13	1923.29	2128.64							
	GPx4	Glutathione peroxidase 4	scaffold0196_285943	CL355Contig4		Cytoplasm	AT1G63460	13.74	13.67	0.00							
	GPx5	Glutathione peroxidase 5	scaffold0959_355995	CL449Contig1	miRNAn3, in progress	Chloroplast	AT2G31570	1332.98	1728.54	1495.92			48.67	76.00	65.00		
	GPx6	Glutathione peroxidase 6	scaffold0196_372488	CL1Contig3643		Mitochondrion	AT4G11600	6316.13	3043.88	3565.20	-1.05						
	GPx7	Glutathione peroxidase 7	scaffold0564_591898	CL354Contig7		Chloroplast, Mitochondrion	AT2G25080	1301.98	1256.54	1037.75							
	GR1	Glutathione reductase 1	scaffold0981_160045	CL1Contig15684	miR394	Chloroplast, Cytoplasm	AT3G24170	2112.97	1179.79	1359.27	-0.84		458.67	357.67	346.67		
	GR2	Glutathione reductase 2	scaffold0432_673871	CL1Contig1556	miR394	Chloroplast, Cytoplasm	AT3G24170	1764.34	2213.28	2437.79			443.67	417.67	411.00		
	GR3	Glutathione reductase 3	scaffold0168_1741285	CL1637Contig2		Chloroplast	AT3G54660	137.82	156.17	129.95							
	MDHAR1	Monodehydroascorbate reductase 1	scaffold3218_2988	CL1Contig7966	miRNAn5, in progress	Chloroplast	AT3G52880	131.22	270.40	266.36	1.04		35.33	64.00	51.33		
	MDHAR2	Monodehydroascorbate reductase 2	scaffold0031_1829426	CL1Contig16376	phased siRNA n4	Cytoplasm	AT3G52880	321.19	660.98	714.20	1.04		67.33	155.00	146.00		
	MDHAR3	Monodehydroascorbate reductase 3	scaffold0764_546000	CL1250Contig6	miRNAn6, in progress	Chloroplast	AT1G63940	77.67	96.70	91.67			13.00	20.00	23.00		
	MDHAR4	Monodehydroascorbate reductase 4	scaffold0980_3829	CL1716Contig1		Chloroplast	AT3G27820	132.89	173.38	209.96							
	MDHAR5	Monodehydroascorbate reductase 5	scaffold0021_379549	CL1716Contig2		Chloroplast	AT3G27820	126.29	101.26	121.78							
	DHAR1	Dehydroascorbate reductase 1	scaffold0276_83160	CL1Contig21106		Cytoplasm	AT1G75270	0.00	8.26	35.76		2.11					
	DHAR2	Dehydroascorbate reductase 2	scaffold0304_978157	CL1Contig478		Cytoplasm	AT1G75270	1747.46	1118.90	1210.71							
	DHAR3	Dehydroascorbate reductase 3	scaffold0476_167817	CL4186Contig3		Chloroplast	AT5G16710	675.26	1043.94	953.78	0.63						
	MSRA1	Methionine sulfoxide reductase A1	scaffold0525_697027	CL432Contig2		-	AT5G61640	681.77	402.76	587.71	-0.76						
	MSRA2	Methionine sulfoxide reductase A2	scaffold0426_978829	CL372Contig4	miRNAn4, in progress	Chloroplast, Nucleus	AT4G25130	3381.54	4348.14	5874.10			1534.00	2072.00	2773.67		
	MSRA2	Methionine sulfoxide reductase A2	scaffold0426_978829	CL372Contig4	phased siRNA n5	Chloroplast, Nucleus	AT4G25130	3381.54	4348.14	5874.10			1720.00	1978.00	2563.67		
	MSRA3	Methionine sulfoxide reductase A3	scaffold0253_168423	CL372Contig3	phased siRNA n6	Nucleus	AT4G25130	2481.01	2217.90	2251.39			217.00	221.00	254.00		
	MSRA4	Methionine sulfoxide reductase A4	scaffold0164_1113953	CL865Contig4		Chloroplast	AT2G18030	323.13	588.07	634.69	0.86						
	MSRA5	Methionine sulfoxide reductase A5	scaffold0100_1344004	CL865Contig2		Chloroplast	AT2G18030	207.41	228.01	228.78							
	MSRB1	Methionine sulfoxide reductase B1	scaffold1541_45658	CL2749Contig1		Chloroplast	AT4G21860	1635.80	880.01	1299.77	-0.89						
	MSRB2	Methionine sulfoxide reductase B2	scaffold0458_602721	CL450Contig3		Cytoplasm	AT4G21860	1109.83	289.73	520.71	-1.94						
	Prx2C1	Peroxiredoxin 2-Cys 1	scaffold0583_705071	CL124Contig1	phased siRNA n7	Chloroplast	AT5G06290	483.65	204.85	304.54	-1.24		177.33	78.00	132.67	-1.51	
	Prx2C2	Peroxiredoxin 2-Cys 2	scaffold0005_99542	CL124Contig2		Chloroplast	AT3G11630	1117.44	449.93	650.92	-1.31						
	Prx2F	Peroxiredoxin-2F 1	scaffold0139_989815	CL1Contig10244		Mitochondrion	AT3G06050	641.45	695.46	1004.36							
	Prx2B1	Peroxiredoxin-2B 1	scaffold0198_1471103	CL1Contig10722		Cytoplasm	AT1G65980	2479.30	1368.08	1871.18	-0.86						
	Prx2B2	Peroxiredoxin-2B 2	scaffold0744_327281	CL1Contig11458		Cytoplasm	AT1G65980	1411.46	4329.61	5229.97	1.62						
	Prx2E	Peroxiredoxin-2E 1	scaffold0360_1177151	CL1Contig5964	phased siRNA n8	Chloroplast	AT3G52960	1043.77	589.67	1213.50	-0.82	1.04	2.67	4.00	8.00		
	PrxQ	Peroxiredoxin Q	scaffold0272_190240	CL3172Contig3		Nucleus	AT3G26060	18.21	35.76	31.82							
	NTR1	NADPH-dependent thioredoxin reductase 1	scaffold0536_346249	CL2806Contig1		Cytoplasm	AT4G35460	965.89	1527.58	1653.27							
	NTR2	NADPH-dependent thioredoxin reductase 2	scaffold0443_30522	CL1Contig6711		Mitochondrion, Chloroplast	AT2G41680	73.68	130.44	96.54							
	NTR3	NADPH-dependent thioredoxin reductase 3	scaffold0965_30248	CL2806Contig1		Cytoplasm	AT4G35460	965.89	1527.58	1653.27							
	GSTU1	Glutathione S-transferase U1	scaffold0062_205328	CL11152Contig1		Cytoplasm	AT1G74590	72.09	103.52	106.48							
	GSTU2	Glutathione S-transferase U2	scaffold2783_15738	CL1Contig2482		Cytoplasm	AT1G17180	22.86	19.32	11.73							
	GSTU3	Glutathione S-transferase U3	scaffold0135_1578719	CL1Contig2381		Cytoplasm	AT1G78380	1826.48	1270.54	1708.45							
	GSTU4	Glutathione S-transferase U4	scaffold0224_135165	CL1Contig2501		Cytoplasm	AT2G29420	0.00	0.00	43.56		2.33					
	GSTU5	Glutathione S-transferase U5	scaffold0862_408658	CL1Contig260		Chloroplast, Cytoplasm	AT1G10370	11.02	23.99	96.68		2.01					
	GSTU6	Glutathione S-transferase U6	scaffold0397_270313	CL1Contig636		Chloroplast, Cytoplasm	AT1G10370	190.20	60.82	121.68	-1.64						
	GSTU7	Glutathione S-transferase U7	scaffold0853_295003	CL3344Contig1		Cytoplasm	AT1G74590	22.96	11.23	21.64							
	GSTU8	Glutathione S-transferase U8	scaffold0702_452766	CL3344Contig2		Cytoplasm	AT1G74590	12.38	31.34	39.84							
	GSTU9	Glutathione S-transferase U9	scaffold0620_625772	CL6613Contig1		Cytoplasm	AT1G78380	32.88	17.00	49.22							
	GSTU10	Glutathione S-transferase U10	scaffold0168_1001290	CL9699Contig1		Cytoplasm	AT3G09270	22.46	17.38	0.00							
	GSTU11	Glutathione S-transferase U11	scaffold0702_454607	CL3344Contig2		Cytoplasm	AT1G74590	12.38	31.34	39.84							
	GSTT1	Glutathione S-transferase T1	scaffold0196_425216	CL1Contig10840		Cytoplasm	AT5G41210	23.37	26.03	22.24							
	GSTT2	Glutathione S-transferase T2	scaffold1121_98799	CL1Contig12924		Cytoplasm	AT5G41210	87.81	118.81	97.98							
	GSTL1	Glutathione S-transferase L1	scaffold0086_1397828	CL1Contig11567		Cytoplasm	AT5G02790	124.11	95.66	127.53							
	GSTL2	Glutathione S-transferase L2	scaffold0086_1410089	CL1Contig9132		Cytoplasm	AT5G02790	150.15	78.76	117.90	-0.93						
	GSTL3	Glutathione S-transferase L3	scaffold2112_30802	CL3924Contig3		Chloroplast	AT3G55040	27.95	19.32	20.32							
	GSTF1	Glutathione S-transferase F1	scaffold0434_14371	CL1Contig12879	phased siRNA n9	Cytoplasm	AT2G30860	4968.29	2310.96	3892.58	-1.10		983.67	470.67	878.67	-1.46	
	GSTF2	Glutathione S-transferase F2	scaffold0197_1256978	CL1Contig20649		Cytoplasm	AT1G02930	153.39	580.67	551.18	1.92						
	GSTF3	Glutathione S-transferase F3	scaffold0742_546080	CL5812Contig1		Cytoplasm	AT2G47730	14.45	26.27	19.63							
	GSTF4	Glutathione S-transferase F4	scaffold0944_303940	CL8413Contig1		Cytoplasm	AT3G62760	15.61	18.94	79.07							
	GSTZ1	Glutathione S-transferase Z1	scaffold3758_4631	CL1Contig18412		Cytoplasm	AT2G02390	77.03	27.06	44.41	-1.51						
	GSTTC	Glutathione S-transferase TCHQD	scaffold0073_65353	CL8458Contig2		Cytoplasm	AT1G77290	167.25	237.20	211.60							
	MGST	Glutathione S-transferase M	scaffold0356_53754	CL1Contig10609		Membrane	At1g65820	1201.10	498.74	641.27	-1.27						
	Grx4C1	Glutaredoxin 4CxC1	scaffold0091_2290103	CL15621Contig1	phased siRNA n10	Nucleus	AT5G58530	255.68	429.09	643.00			60.00	98.33	112.00		
	Grx4C2	Glutaredoxin 4CxC2	scaffold1092_15278	CL7844Contig1		Nucleus	AT3G57070	52.14	9.96	0.00	-2.39						
	GrxR1	Glutaredoxin ROXY1	scaffold0630_314223	CL17067Contig1		Cytoplasm, Chloroplast	AT1G28480	110.76	64.05	26.55							
	GrxR2	Glutaredoxin ROXY2	scaffold0610_362800	CL2864Contig3		Chloroplast	AT1G28480	18.29	75.25	37.09	2.04						
	GrxR3	Glutaredoxin ROXY3	scaffold4986_4172	CL11128Contig1		Chloroplast	AT1G28480	602.32	2767.72	2674.66	2.20						
	GrxR4	Glutaredoxin ROXY4	scaffold0043_1751365	CL3091Contig4		Chloroplast	AT4G33040	5109.21	4356.96	9520.19		1.13					
	GrxC1	Glutaredoxin GrxC1	scaffold0388_37595	CL139Contig10		Chloroplast	AT5G63030	508.61	814.25	879.87	0.68						
	GrxC2	Glutaredoxin GrxC2	scaffold0132_615791	CL1Contig7489		Chloroplast	AT5G40370	4713.64	2080.40	3350.53	-1.18						
	GrxC3	Glutaredoxin GrxC3	scaffold0893_145933	CL5179Contig1		Chloroplast	AT1G77370	336.49	195.74	229.92	-0.78						
	GrxC4	Glutaredoxin GrxC4	scaffold0398_733626	CL5179Contig3		Plastid	AT1G77370	67.10	28.83	42.91	-1.22						
	GrxC5	Glutaredoxin GrxC5	scaffold1097_168708	CL1118Contig1		Chloroplast	AT5G20500	534.52	256.24	377.19	-1.06						
	GrxC6	Glutaredoxin GrxC6	scaffold1440_108116	CL1Contig17682		Chloroplast	AT4G28730	43.11	92.13	72.48	1.10						
	GrxC7	Glutaredoxin GrxC7	scaffold1012_295953	CL1Contig20255		Chloroplast	AT4G28730	45.57	98.03	90.92	1.11						
	GrxC8	Glutaredoxin GrxC8	scaffold1078_119123	CL139Contig7		Chloroplast	AT5G63030	855.55	335.16	426.07	-1.35						
	GrxS1	Glutaredoxin GrxS1	scaffold0733_502106	CL1153Contig1		Chloroplast	AT3G15660	1432.75	1438.12	1584.33							
	GrxS2	Glutaredoxin GrxS2	scaffold1428_54601	CL1153Contig2		Chloroplast	AT3G15660	826.80	1189.51	1110.62							
	GrxS3	Glutaredoxin GrxS3	scaffold1804_58897	CL1Contig18246		Cytoplasm, Chloroplast	AT4G04950	3660.93	1785.69	1920.69	-1.04						
	GrxS4	Glutaredoxin GrxS4	scaffold0774_520283	CL316Contig1		Chloroplast	AT3G54900	682.78	524.05	810.08							
	GrxS5	Glutaredoxin GrxS5	scaffold1206_201280	CL316Contig7		Chloroplast	AT3G54900	750.87	749.80	904.96							
	GrxS6	Glutaredoxin GrxS6	scaffold0016_414036	CL4496Contig1		Chloroplast	AT2G38270	368.86	558.63	545.09	0.60						
	TrxH1	Thioredoxin H1	scaffold0052_812663	CL1Contig41		Cytoplasm	AT5G39950	370.96	181.88	253.43	-1.03						
	TrxH2	Thioredoxin H2	scaffold0378_800539	CL1254Contig7		Cytoplasm, Nucleus	AT3G08710	457.73	892.77	909.81	0.96						
	TrxH3	Thioredoxin H3	scaffold0470_330341	CL1254Contig8		Cytoplasm	AT3G08710	487.35	788.31	734.13	0.69						
	TrxH4	Thioredoxin H4	scaffold0893_84684	CL1Contig10487		Cytoplasm	AT3G51030	1878.28	3256.30	3326.63	0.79						
	TrxH5	Thioredoxin H5	scaffold0029_1224586	CL1Contig10790		Cytoplasm	AT3G51030	30280.75	17358.36	24221.47	-0.80						
	TrxH6	Thioredoxin H6	scaffold0008_512244	CL1Contig9123		Cytoplasm	AT3G51030	7359.20	2967.38	5148.08	-1.31						
	TrxA1	Thioredoxin ACHT1	scaffold0794_475278	CL163Contig2		Chloroplast	AT1G08570	295.76	315.83	240.22							
	TrxA2	Thioredoxin ACHT2	scaffold0163_936086	CL1730Contig4		Chloroplast	AT4G29670	19.96	8.93	0.00							
	TrxA3	Thioredoxin ACHT3	scaffold0894_342753	CL1730Contig5		Plastid	AT4G29670	308.50	372.28	393.05							
	TrxA4	Thioredoxin ACHT4	scaffold0776_350933	CL163Contig8		Chloroplast	AT1G08570	2622.86	2620.78	2410.88							
	TrxA5	Thioredoxin ACHT5	scaffold0054_2161171	CL1Contig21402	phased siRNA n11	Chloroplast	AT1G08570	2291.97	2475.47	2423.06			197.00	201.33	257.67		
	TrxA6	Thioredoxin ACHT6	scaffold1735_35575	CL1Contig13339		Chloroplast	At2g33270	211.72	252.35	245.29							
	TrxS12	Thioredoxin CxxS12	scaffold0683_226073	CL1Contig15370		Cytoplasm, Chloroplast	AT1G11530	1286.30	257.30	531.86	-2.32	1.05					
	TrxS13	Thioredoxin CxxS13	scaffold0616_558335	CL3418Contig1		Cytoplasm	AT1G11530	2821.90	378.57	809.06	-2.90	1.10					
	TrxF1	Thioredoxin F1	scaffold0868_251302	CL1Contig16697	phased siRNA n12	Chloroplast	AT5G16400	11.39	26.41	13.70			1.33	3.67	1.67		
	TrxF2	Thioredoxin F2	scaffold0177_327545	CL1Contig2732		Chloroplast	AT5G16400	211.50	373.70	430.57	0.82						
	TrxM1	Thioredoxin M1	scaffold1024_219828	CL1Contig18661		Chloroplast	AT1G03680	1467.22	1645.72	1633.19							
	TrxM2	Thioredoxin M2	scaffold0389_250593	CL1Contig6171		Chloroplast	AT1G03680	744.88	1136.47	1092.54	0.61						
	TrxM3	Thioredoxin M3	scaffold0079_1290430	CL5346Contig1	phased siRNA n13	Chloroplast	AT2G15570	1412.94	1613.28	2077.85			349.67	410.33	464.00		
	TrxW1	Thioredoxin WCRKC1	scaffold2423_24886	CL1Contig5981		Chloroplast	AT5G06690	629.26	350.16	331.81	-0.85						
	TrxW2	Thioredoxin WCRKC2	scaffold0160_1197841	CL6528Contig1		Chloroplast, Nucleus	AT5G04260	306.01	374.38	358.79							
	TrxO	Thioredoxin O	scaffold0032_237207	CL2392Contig2		Chloroplast, Nucleus	AT2G35010	2187.01	3067.87	3030.19							
	TrxWC	Thioredoxin WCGVC	scaffold0251_1319405	CL2651Contig3		Cytoplasm, Nucleus	AT3G53220	236.77	132.47	189.29	-0.84						
	TrxZ1	Thioredoxin Z1	scaffold1019_111525	CL4085Contig3		Chloroplast	AT3G06730	79.20	39.05	98.68	-1.02						
	TrxHC	Thioredoxin HCF164	scaffold0253_868182	CL4497Contig1		Chloroplast	AT4G37200	240.35	233.79	315.57							
	TrxC1	Thioredoxin Clot1	scaffold1466_8923	CL1Contig10589		-	AT5G42850	8772.81	4961.45	5843.33	-0.82						
	TrxY1	Thioredoxin Y1	scaffold0168_67655	CL782Contig3		Chloroplast	AT1G43560	224.63	252.80	281.12							
	TrxY2	Thioredoxin Y2	scaffold0318_1135002	CL782Contig4		Chloroplast	AT1G43560	2096.99	2115.35	2269.63							
	Nrx1	Nucleoredoxin 1	scaffold0866_92082	CL322Contig5		Nucleus	At1g60420	273.66	89.34	108.83	-1.61						
	Nrx2	Nucleoredoxin 2	scaffold1000_300919	CL1753Contig2		Cytoplasm	At1g60420	4061.48	1949.08	2287.15	-1.06						
	Nrx3	Nucleoredoxin 3	scaffold0574_591762	CL322Contig2		Cytoplasm. Nucleus	At1g60420	235.66	252.41	195.80							
	Nrx4	Nucleoredoxin 4	scaffold0198_911190	CL4868Contig1		Cytoplasm	At4g31240	1642.27	2713.15	2997.32	0.72						
Ascorbate biosynthesis	VTC21	GDP-L-galactose phosphorylase 1	scaffold0434_787620	CL1Contig10550		Chloroplast	AT4G26850	3488.93	12239.95	15303.38	1.81						
	VTC22	GDP-L-galactose phosphorylase 2	scaffold0552_511240	CL1Contig3683		Chloroplast, Cytoplasm	AT4G26850	16301.27	9058.02	15106.25	-0.85						
	VTC11	GDP-D-mannose pyrophosphorylase 1	scaffold1151_52122	CL1Contig16141		Cytoplasm	AT2G39770	1524.66	2500.39	2390.09	0.71						
	VTC12	GDP-D-mannose pyrophosphorylase 2	scaffold1247_48513	CL1Contig4217	phased siRNA n14 and n15	Cytoplasm	AT2G39770	640.87	516.34	566.86			226.00	328.00	279.33		
	GME1	GDP-mannose 3,5-epimerase 1	scaffold0239_876734	CL1Contig19546		Cytoplasm	AT5G28840	29.01	61.70	73.41							
	GME2	GDP-mannose 3,5-epimerase 2	scaffold0474_313994	CL1Contig9775		Cytoplasm	AT5G28840	8482.75	12272.27	13400.06							
	GLDH	L-galactono-1,4-lactone dehydrogenase	scaffold0534_479038	CL2271Contig4		Chloroplast	AT3G47930	417.93	399.22	408.70							
	VTC41	Inositol phosphate phosphatase 1	scaffold0295_604670	CL1Contig11896		Cytoplasm	AT3G02870	138.95	66.22	87.38	-1.07						
	VTC42	Inositol phosphate phosphatase 2	scaffold0359_724098	CL1Contig5479		Cytoplasm	AT3G02870	60.61	91.47	74.32							
	GDH1	L-galactose dehydrogenase 1	scaffold1364_78602	CL1895Contig4		Chloroplast, Cytoplasm	AT4G33670	789.73	817.74	943.32							
	GDH2	L-galactose dehydrogenase 2	scaffold1364_29743	CL1895Contig4		Chloroplast, Cytoplasm	AT4G33670	789.73	817.74	943.32							
	GDH3	L-galactose dehydrogenase 3	scaffold0200_1577729	CL1895Contig6		Chloroplast, Cytoplasm	AT4G33670	72.94	49.42	79.96							
	GULO1	L-gulonolactone oxidase1	scaffold0018_745649	CL7103Contig1		Chloroplast	AT5G11540	393.30	204.35	287.68	-0.94						
	Miox1	Myo-inositol oxygenase 1	scaffold0178_130651	CL234Contig1		Cytoplasm	AT1G14520	315.67	324.02	542.96							
	Miox2	Myo-inositol oxygenase 2	scaffold1991_26122	CL234Contig10	miRNAn8, in progress	Cytoplasm	AT1G14520	175.46	101.98	77.82			35.33	26.67	33.33		
	Miox3	Myo-inositol oxygenase 3	scaffold0137_1767530	CL5668Contig2		Cytoplasm	AT4G26260	311.74	1389.09	753.57	2.16						
Glutathione biosynthesis	GCL1	Glutamate cysteine ligase 1	scaffold0150_1891643	CL1Contig7647		Chloroplast	AT4G23100	851.80	402.25	450.03	-1.08						
	GCL2	Glutamate cysteine ligase 2	scaffold0103_1153497	CL1Contig11386		Chloroplast, Cytoplasm	AT4G23100	907.29	2297.27	1714.37	1.34						
	GS1	Glutathione synthetase 1	scaffold0374_904985	CL660Contig4		Cytoplasm	AT5G27380	283.30	318.93	347.87							
	GS2	Glutathione synthetase 2	scaffold0141_969519	CL660Contig9		Chloroplast	AT5G27380	105.10	199.24	119.24	0.92						
Tocotrienol biosynthesis	VTE31	MPBQ/MSBQ methyltransferase 1	scaffold1284_33738	CL1Contig10053		Chloroplast	AT3G63410	2856.56	1867.10	2033.71							
	VTE32	MPBQ/MSBQ methyltransferase 2	scaffold0576_731692	CL1Contig5786		Chloroplast	AT3G63410	2611.24	911.67	1274.35	-1.52						
	VTE33	MPBQ/MSBQ methyltransferase 3	scaffold0031_3305736	CL5665Contig1	miRNAn7, in progress	Chloroplast	AT3G63410	285.43	267.35	316.95			68.33	66.00	85.67		
	VTE2	Homogentisate phytyltransferase	scaffold0729_135602	CL4970Contig3		Plastid membrane	AT2G18950	3228.13	1246.29	1350.45	-1.37						
	VTE1	Tocopherol cyclase	scaffold0432_926217	CL4101Contig2		Chloroplast	AT4G32770	999.99	912.03	869.30							
	VTE4	Tocopherol gamma-methyltransferase	scaffold0168_1042895	CL1409Contig4	phased siRNA n16	Chloroplast	AT1G64970	17.36	497.89	259.84	4.84		1.00	73.33	35.00	5.69	4.51

0 | 200 | 1000 | 5000 | 15000

Figure 17. Supplementary data 1

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Additional study beyond the published data

Latex ROS scavenging system is complex. To figure out the main ROS-scavenging genes among 161 latex expressing genes is the key point for achieving the prospect of gene modification and molecular genetic marker. There was a transcriptome database generated by our group from 454 RNA sequencing for 6 *Hevea* tissues (bark, embryo, latex, leaf, reproductive tissues and root) in PB260. Unfortunately, it was not deep enough to make statistical analysis but important to check the redox-related genes' expression in different tissues.

As for the studied PB260 clone, a TPD susceptible plant, there was RNAseq data from tolerant clone SP217. The limitation of this data was that the sample of healthy tree after ethephon treatment was missing because of low quality of RNA. SP217 was tapped every day while PB260 was every 2 days. Because of TPD tolerant capacity, the TPD trees of SP217 was induced only by higher-frequency of tapping. Consequently, SP217 and PB260 were not sampled in the same condition. SP217 was sampled under heavier stress. To avoid the weakness of those data confusing the readers, we did not put the gene expression data from SP217 and different tissues of PB260 in the published paper of this chapter. However, studying the expression data of redox-related genes latex of SP217 is a good reference for the future experiment design in future.

Redox-related genes' expression in different tissues

All this latex expressing redox related genes were not latex specific expressing genes. We could find some reads from other tissues (Figure 14). However, there were 22 genes expressed abundantly in latex comparing with in other tissues: *HbPPO1*, *HbGPx6*, *HbMSRA2*, *HbMSRB2*, *HbPRX2B2*, *HbMGST*, *HbGrxC2*, 13 *HbTrx*s, *HbVTE2* and *HbVTC22*. Peroxidases expressed at very low level in latex, with only several reads detected (Figure 14). By contrast, they were expressed at very high level in some other tissues: 1162 and 559 reads of *HbPx1* detected in bark and root respectively as well as 788, 1991 and 536 reads of HbPx5 detected in bark, embryo and root respectively.

Redox-related genes' expression in SP217

Reads per kilobase per million mapped reeds (RPKM) were presented as the expression of SP217 and PB260 (Figure 15). The comparison between SP217 and PB260 was made *via* T-test. The effect on these two different clones under different tapping frequencies was presented in Figure 16 only the P-value was below 5%. There were two comparisons between the two clones: healthy tree after water treatment (W/H) and TPD tree after ethephon treatment (ET/TPD). The expression patterns of redox related genes in these two clones were very different (Figure 15). Indeed, 11 and 12 redox related genes were expressed at higher and lower levels respectively both in healthy and TPD tree of SP217. Furthermore, 17 and 21 redox related genes were expressed at higher and lower levels respectively in healthy tree of SP217. Finally, 8 and 6 redox related genes were expressed at higher and lower respectively in TPD tree of SP217. There were 3 more peroxidase genes expressed in the latex of SP217: *HbPx19*, *HbPx22* and *HbPx53*. *HbPx22* and *HbPx53* were localized in extracellular space and *HbPx19* had unknown localization.

Gene	Enzyme	Bark	Embryo	Latex	Leaf	Reprod	Root
PX1	Peroxidase 1	1162	382	5	363	193	559
PX2	Peroxidase 2	2	15	0	1	14	5
PX3	Peroxidase 3	37	30	0	41	51	21
PX4	Peroxidase 4	1	1	10	0	69	10
PX5	Peroxidase 5	788	1997	6	145	94	536
PX6	Peroxidase 6	140	334	0	3	37	3
PX7	Peroxidase 7	19	12	1	3	9	4
PX19	Peroxidase 19	29	14	0	11	25	207
PX22	Peroxidase 22	28	5	0	11	0	22
PX53	Peroxidase 53	3	3	0	0	2	2
CAT1	Catalase 1	86	54	21	48	33	193
CAT2	Catalase 2	71	205	17	26	71	158
CAT3	Catalase 3	8	6	14	24	4	22
CuZnSOD1	Cu/Zn superoxide dismutase 1	35	18	31	67	25	18
CuZnSOD2	Cu/Zn superoxide dismutase 2	24	31	1	57	29	19
CuZnSOD3	Cu/Zn superoxide dismutase 3	51	85	15	111	88	54
CuZnSOD4	Cu/Zn superoxide dismutase 4	6	0	27	2	1	1
MnSOD1	Mn superoxide dismutase 1	68	222	32	64	35	159
MnSOD2	Mn superoxide dismutase 2	55	102	28	60	74	84
APX1	Ascorbate peroxidase 1	366	701	34	352	81	550
APX2	Ascorbate peroxidase 2	183	422	7	32	36	149
APX3	Ascorbate peroxidase 3	15	7	2	5	5	8
APX4	Ascorbate peroxidase 4	119	162	32	65	35	160
APX5	Ascorbate peroxidase 5	17	27	9	73	14	52
GPX1	Glutathione Peroxidase 1	7	6	4	33	5	1
GPX2	Glutathione Peroxidase 2	1	0	7	3	5	2
GPX3	Glutathione peroxidase 3	24	31	33	25	24	20
GPX4	Glutathione peroxidase 4	2	7	0	1	1	9
GPX5	Glutathione peroxidase 5	19	21	109	193	27	10
GPX6	Glutathione peroxidase 6	82	45	162	125	70	123
GPX7	Glutathione peroxidase 7	23	15	31	87	15	33
GR1	Glutathione reductase 1	59	56	32	43	82	70
GR2	Glutathione reductase 2	19	41	23	7	43	25
GR3	Glutathione reductase 3	17	20	0	2	38	25
MDHAR1	Monodehydroascorbate reductase 1	96	196	1	25	95	306
MDHAR2	Monodehydroascorbate reductase 2	134	125	5	72	177	252
MDHAR3	Monodehydroascorbate reductase 3	5	4	0	7	9	11
MDHAR4	Monodehydroascorbate reductase 4	30	6	1	17	28	21
MDHAR5	Monodehydroascorbate reductase 5	6	5	0	1	7	1
DHAR1	Dehydroascorbate reductase 1	1	0	1	1	2	0
DHAR2	Dehydroascorbate reductase 2	41	83	29	57	19	70
DHAR3	Dehydroascorbate reductase 3	17	14	8	20	5	9
MSRA1	Methionine sulfoxide reductase A1	19	9	34	181	46	20
MSRA2	Methionine sulfoxide reductase A2	12	2	115	46	30	14
MSRA3	Methionine sulfoxide reductase A3	12	7	61	63	33	5
MSRA4	Methionine sulfoxide reductase A4	11	16	2	13	3	9
MSRA5	Methionine sulfoxide reductase A5	7	9	2	5	2	5
MSRB1	Methionine sulfoxide reductase B1	9	0	22	76	11	2
MSRB2	Methionine sulfoxide reductase B2	6	7	184	51	34	1
PRX2C1	Peroxiredoxin 2-Cys 1	93	68	4	219	23	83
PRX2C2	Peroxiredoxin 2-Cys 2	30	13	21	129	9	32
PRX2F	Peroxiredoxin-2F 1	9	14	13	27	45	19
PRX2B1	Peroxiredoxin-2B 1	266	383	192	180	63	202
PRX2B2	Peroxiredoxin-2B 2	25	54	76	37	20	25
PRX2E	Peroxiredoxin-2E 1	8	24	14	33	32	18
PRXQ	Peroxiredoxin Q	6	0	0	76	4	5
GSTU1	Glutathione S-transferase U1	5	1	0	3	3	0
GSTU2	Glutathione S-transferase U2	2	2	0	0	4	0
GSTU3	Glutathione S-transferase U3	240	327	142	101	114	206
GSTU4	Glutathione S-transferase U4	21	3	0	1	1	8
GSTU5	Glutathione S-transferase U5	77	72	0	65	45	14
GSTU6	Glutathione S-transferase U6	12	23	8	56	5	10
GSTU7	Glutathione S-transferase U7	0	51	0	3	1	31
GSTU8	Glutathione S-transferase U8	0	3	1	0	0	6
GSTU9	Glutathione S-transferase U9	5	9	0	1	9	18
GSTU10	Glutathione S-transferase U10	1	6	0	9	1	0
GSTU11	Glutathione S-transferase U11	0	3	1	0	0	6
GSTT1	Glutathione S-transferase T1	0	1	2	0	0	0
GSTT2	Glutathione S-transferase T2	33	34	0	28	11	23
GSTL1	Glutathione S-transferase L1	10	4	4	3	16	0
GSTL2	Glutathione S-transferase L2	56	176	1	212	58	70
GSTL3	Glutathione S-transferase L3	0	0	0	2	0	3
GSTF1	Glutathione S-transferase F1	87	83	118	227	93	254
GSTF2	Glutathione S-transferase F2	10	87	7	72	80	87
GSTF3	Glutathione S-transferase F3	2	1	0	44	1	3
GSTF4	Glutathione S-transferase F4	1	13	0	0	8	3
GSTZ	Glutathione S-transferase Z1	34	18	4	28	19	40
GSTTC	Glutathione S-transferase TCHQD	5	3	1	0	4	2
MGST	Glutathione S-transferase M	8	62	76	69	32	34
NTR1	NADPH-dependent thioredoxin reductase 1	12	38	11	17	15	28
NTR2	NADPH-dependent thioredoxin reductase 2	11	9	0	1	7	10
NTR3	NADPH-dependent thioredoxin reductase 3	12	38	11	17	15	28
		0	50	200	1000	1500	

Gene	Enzyme	Bark	Embryo	Latex	Leaf	Reprod	Root
Rboh1	Respiratory burst oxidase homolog 1	13	15	0	0	12	57
Rboh2	Respiratory burst oxidase homolog 2	0	4	4	0	5	3
PPO1	Polyphenol oxidase 1	0	1	191	0	30	2
PPO2	Polyphenol oxidase 2	0	13	0	0	2	2
GRX4C1	Glutaredoxin 4CxxC1	0	2	1	1	2	0
GRX4C2	Glutaredoxin 4CxxC2	1	3	1	3	22	0
GRXR1	Glutaredoxin ROXY1	0	0	1	0	2	2
GRXR2	Glutaredoxin ROXY2	31	6	1	10	34	19
GRXR3	Glutaredoxin ROXY3	0	0	5	0	6	1
GRXR4	Glutaredoxin ROXY4	0	1	26	0	54	1
GRXC1	Glutaredoxin GrxC1	1	1	19	20	17	0
GRXC2	Glutaredoxin GrxC2	80	292	551	502	72	46
GRXC3	Glutaredoxin GrxC3	1	1	20	6	3	1
GRXC4	Glutaredoxin GrxC4	1	3	4	2	5	3
GRXC5	Glutaredoxin GrxC5	8	14	52	19	26	16
GRXC6	Glutaredoxin GrxC6	0	0	0	5	3	2
GRXC7	Glutaredoxin GrxC7	3	2	2	10	7	1
GRXC8	Glutaredoxin GrxC8	28	29	21	23	58	32
GRXS1	Glutaredoxin GrxS1	19	16	25	36	8	8
GRXS2	Glutaredoxin GrxS2	11	15	13	29	29	9
GRXS3	Glutaredoxin GrxS3	96	49	82	12	43	125
GRXS4	Glutaredoxin GrxS4	61	45	46	59	41	34
GRXS5	Glutaredoxin GrxS5	9	5	15	4	9	3
GRXS6	Glutaredoxin GrxS6	5	10	4	23	15	15
TRXH1	Thioredoxin H1	25	23	172	80	111	9
TRXH2	Thioredoxin H2	1	2	4	11	2	2
TRXH3	Thioredoxin H3	16	17	23	27	11	10
TRXH4	Thioredoxin H4	9	21	701	74	8	12
TRXH5	Thioredoxin H5	57	52	2510	273	79	17
TRXH6	Thioredoxin H6	1024	760	826	816	177	208
TRXA1	Thioredoxin ACHT1	28	2	5	1	10	16
TRXA2	Thioredoxin ACHT2	10	5	2	21	6	8
TRXA3	Thioredoxin ACHT3	20	11	15	16	19	18
TRXA4	Thioredoxin ACHT4	9	16	53	9	16	9
TRXA5	Thioredoxin ACHT5	66	14	56	62	61	57
TRXA6	Thioredoxin ACHT6	1	0	1	0	10	3
TRXS12	Thioredoxin CxxS12	4	2	84	10	75	5
TRXS13	Thioredoxin CxxS13	0	0	100	0	0	1
TRXF1	Thioredoxin F1	17	3	0	63	20	6
TRXF2	Thioredoxin F2	22	4	7	45	29	16
TRXM1	Thioredoxin M1	11	10	29	25	31	14
TRXM2	Thioredoxin M2	9	8	14	13	13	14
TRXM3	Thioredoxin M3	1	2	28	7	14	5
TRXW1	Thioredoxin WCRKC1	8	11	11	58	16	8
TRXW2	Thioredoxin WCRKC2	1	10	7	3	3	15
TRXO	Thioredoxin O	14	24	31	14	30	18
TRXWC	Thioredoxin WCGVC	0	4	71	12	1	1
TRXZ	Thioredoxin Z1	4	1	3	7	4	0
TRXHC	Thioredoxin HCF164	16	5	1	30	7	14
TRXC	Thioredoxin Clot1	6	21	567	102	5	8
TRXY1	Thioredoxin Y1	6	3	9	12	12	1
TRXY2	Thioredoxin Y2	22	16	35	100	26	15
NRX1	Nucleoredoxin 1	32	17	7	4	4	103
NRX2	Nucleoredoxin 2	8	33	34	4	39	46
NRX3	Nucleoredoxin 3	32	27	2	11	26	35
NRX4	Nucleoredoxin 4	8	3	29	9	7	8
VTC21	GDP-L-galactose phosphorylase 1	549	134	31	229	190	446
VTC22	GDP-L-galactose phosphorylase 2	94	8	306	42	82	45
VTC11	GDP-D-mannose pyrophosphorylase 1	148	70	13	10	19	120
VTC12	GDP-D-mannose pyrophosphorylase 2	136	89	12	11	45	261
GME1	GDP-mannose 3,5-epimerase 1	977	176	1	94	90	410
GME2	GDP-mannose 3,5-epimerase 2	112	87	113	22	35	138
GLDH	L-galactono-1,4-lactone dehydrogenase	26	15	10	10	20	28
VTC41	Inositol phosphate phosphatase 1	6	15	1	0	10	18
VTC42	Inositol phosphate phosphatase 2	20	23	1	11	62	32
GDH1	L-galactose dehydrogenase 1	18	9	22	15	15	32
GDH2	L-galactose dehydrogenase 2	18	9	22	15	15	32
GDH3	L-galactose dehydrogenase 3	0	0	2	1	0	0
GLO	L-gulonolactone oxidase1	4	10	10	1	6	4
MIOX1	Myo-inositol oxygenase 1	102	0	5	0	1	0
MIOX2	Myo-inositol oxygenase 2	46	59	3	2	22	16
MIOX3	Myo-inositol oxygenase 3	7	0	3	1	8	34
GCL1	Glutamate cysteine ligase 1	31	24	9	3	24	36
GCL2	Glutamate cysteine ligase 2	53	32	13	9	48	134
GS1	Glutathione synthetase 1	8	4	2	7	4	2
GS2	Glutathione synthetase 2	10	12	0	0	13	49
VTE31	MPBQ/MSBQ methyltransferase 1	20	14	37	18	19	35
VTE32	MPBQ/MSBQ methyltransferase 2	61	28	69	107	36	68
VTE33	MPBQ/MSBQ methyltransferase 3	8	7	6	17	6	9
VTE2	Homogentisate phytyltransferase	2	6	37	0	7	1
VTE1	Tocopherol cyclase	1	4	9	2	10	4
VTE4	Tocopherol gamma-methyltransferase	8	76	1	17	21	31
		0	50	200	1000	1500	

Figure 18. Redox-related genes' expression in different tissues by 454 RNAseq database.

Gene	SP217		PB260		SP217/PB260 Effect	
	W/H	ET/TPD	W/H	ET/TPD	W/H	ET/TPD
Px1	8.39	2.39	2.67	0.47	1.65	2.33
Px2	1.44	1.46	0.37	1.18		
Px3	0.43	0.65	0.63	0.77		
Px4	7.37	5.54	6.70	6.46		
Px5	0.23	1.11	2.20	1.17	-3.25	
Px6	0.43	5.40	0.63	4.72		
Px7	0.49	0.54	0.88	0.41	-0.84	
Px19	0.79	0.74	0.00	0.00		
Px22	0.73	0.00	0.00	0.00		
Px53	1.27	0.00	0.00	0.00		
Cat1	61.24	163.89	64.31	100.85		
Cat2	13.58	13.94	17.01	10.60		
Cat3	69.32	57.32	73.76	73.50		
CuZnSOD1	62.10	40.91	68.14	58.28		-0.51
CuZnSOD2	5.48	10.51	8.25	10.86		
CuZnSOD3	13.02	10.51	14.66	13.21		
CuZnSOD4	223.97	130.09	26.40	5.27	3.08	4.62
MnSOD1	46.26	44.64	47.62	49.24		
MnSOD2	83.33	75.19	69.64	44.06	0.26	
APx1	17.15	18.09	27.08	19.53	-0.66	
APx2	1.81	0.98	2.70	1.43		
APx3	2.85	5.65	10.48	15.80	-1.88	-1.48
APx4	75.48	108.01	63.97	82.47	0.24	
APx5	19.19	20.88	23.30	25.85		
GPx1	4.13	5.91	4.81	3.76		0.65
GPx2	4.94	4.93	1.76	1.15	1.49	2.10
GPx3	47.69	52.38	57.43	61.91		
GPx4	1.07	1.06	0.72	0.68	0.57	
GPx5	77.43	208.55	52.29	59.17		1.82
GPx6	143.93	96.85	190.02	107.91		
GPx7	25.25	31.98	35.94	29.46	-0.51	
GR1	26.02	18.13	37.76	24.55	-0.54	-0.44
GR2	28.71	54.33	32.09	45.06		
GR3	3.04	4.20	2.68	2.64		
MDHAR1	3.99	5.87	2.89	5.96		
MDHAR2	0.88	5.22	6.77	15.47	-2.94	-1.57
MDHAR3	2.72	3.27	1.48	1.76	0.87	0.89
MDHAR4	2.53	5.40	3.15	5.06	-0.31	
MDHAR5	2.34	3.40	3.04	2.94		0.21
DHAR1	4.81	2.28	0.36	2.05	3.75	
DHAR2	67.99	46.42	67.75	47.37		
DHAR3	11.49	23.83	20.72	29.65	-0.85	-0.32
MSRA1	13.11	9.03	10.92	9.43		
MSRA2	72.01	109.47	97.28	173.40		-0.66
MSRA3	97.12	88.86	76.16	69.89	0.35	
MSRA4	7.06	10.92	8.26	16.37		-0.58
MSRA5	6.23	8.76	6.06	6.70		
MSRB1	36.34	34.51	57.65	46.36	-0.67	
MSRB2	36.35	14.66	36.08	16.80		
Prx2C1	30.80	20.11	14.45	9.24	1.09	1.12
Prx2C2	23.34	13.05	33.46	19.79		
Prx2F	16.08	18.67	21.05	33.31		-0.84
Prx2B1	157.30	104.60	105.81	80.64	0.57	
Prx2B2	82.26	172.30	58.60	219.10	0.49	
Prx2E	36.47	23.10	24.87	29.42		
PrxQ	1.08	1.39	0.62	1.07		
NTR1/3	29.64	53.93	23.96	41.60		
NTR2	0.92	3.21	2.09	2.88		
GSTU1	1.87	2.81	2.56	3.86		
GSTU2	1.81	3.12	1.57	0.84		1.90
GSTU3	64.94	38.21	56.37	53.18		
GSTU4	0.15	3.55	0.08	2.03		
GSTU5	0.18	2.94	0.29	2.44		
GSTU6	14.17	5.11	6.72	4.30	1.08	
GSTU7	2.80	3.66	0.94	0.93	1.57	1.98
GSTU8/11	2.51	4.00	0.69	2.26	1.87	
GSTU9	0.52	0.64	1.27	1.84	-1.28	
GSTU10	0.02	0.08	1.05	0.89	-5.95	-3.53
GSTT1	1.93	2.01	3.59	3.83	-0.89	-0.93
GSTT2	0.86	1.00	2.28	2.47	-1.41	-1.31
GSTL1	93.99	53.85	4.32	4.39	4.44	3.62
GSTL2	3.41	6.06	5.10	3.92		
GSTL3	0.55	0.47	1.34	0.96	-1.28	
GSTF1	233.64	144.44	161.90	127.72		
GSTF2	20.61	41.11	5.29	19.21	1.96	
GSTF3	0.31	0.93	0.58	0.81		
GSTF4	7.69	8.75	0.75	3.84	3.35	
GSTZ1	3.58	3.08	1.95	1.18		1.39
GSTTC	4.03	7.27	6.33	8.11	-0.65	
MGST	73.55	38.37	51.59	27.62	0.51	
	0.00	50.00	300.00	[illegible]		

Gene	SP217		PB260		SP217/PB260 Effect	
	W/H	ET/TPD	W/H	ET/TPD	W/H	ET/TPD
RBOH1	0.14	0.49	0.07	0.10		
RBOH2	0.44	0.96	6.56	5.92	-3.90	
PPO1	94.95	22.02	121.25	13.15		
PPO2	9.89	4.12	12.68	3.19		
Grx4C2	0.60	0.40	1.03	0.29		
GrxR1	2.18	15.01	4.94	1.23		
GrxR2	7.54	24.82	0.83	1.75	3.18	3.82
GrxR3	2.21	24.86	32.55	147.20	-3.88	-2.57
GrxR4	336.05	283.24	225.60	421.15		
GrxC1	21.51	40.12	23.49	40.58		
GrxC2	422.40	187.75	223.74	159.98		
GrxC3	8.92	7.49	18.10	12.40		
GrxC4	1.15	1.05	2.79	1.80	-1.27	
GrxC5	39.71	19.23	31.68	22.59		
GrxC6	1.99	4.83	2.33	3.97		
GrxC7	1.97	5.68	2.15	4.15		
GrxC8	7.90	5.98	13.49	6.79		
GrxS1	49.07	67.35	56.75	63.20		
GrxS2	17.48	32.22	30.38	40.84	-0.80	
GrxS3	69.38	35.78	63.90	33.81		
GrxS4	29.28	25.71	21.28	25.25	0.46	
GrxS5	27.59	36.93	27.36	33.37		
GrxS6	4.14	6.11	10.19	15.22	-1.30	-1.32
TrxH1	54.08	39.34	17.16	12.41	1.66	1.66
TrxH2	19.11	48.65	18.00	36.54		
TrxH3	15.79	28.42	19.61	29.25		
TrxH4	54.89	126.05	79.04	138.11		-0.13
TrxH5	1282.16	922.44	1141.03	929.92		
TrxH6	214.00	172.80	286.99	206.21		
TrxA1	8.63	7.56	7.77	6.53		
TrxA2	0.95	3.03	0.69	0.31	0.46	
TrxA3	3.58	8.33	9.55	12.36	-1.41	
TrxA4	72.22	103.53	69.32	64.79		
TrxA5	29.48	67.12	50.95	55.15		
TrxA6	8.63	15.72	9.69	11.65		
TrxS12	21.38	19.83	50.12	20.10	-1.23	
TrxS13	64.00	21.23	181.28	53.41		
TrxF1	0.15	0.16	0.41	0.51	-1.43	
TrxF2	4.83	5.18	8.74	18.35	-0.86	-1.83
TrxM1	49.41	89.09	55.25	62.53		
TrxM2	9.72	24.81	21.08	31.17	-1.12	
TrxM3	62.48	72.36	54.33	80.83		
TrxW1	3.94	5.29	24.54	13.28	-2.64	
TrxW2	5.39	9.40	9.61	11.41	-0.83	-0.28
TrxO	35.70	76.30	59.46	83.10	-0.74	
TrxWC	7.37	8.24	10.05	8.08		
TrxZ1	3.42	2.61	3.31	4.15		
TrxHC	13.60	20.46	7.71	10.13	0.82	1.01
TrxC1	414.28	307.61	532.10	352.15		
TrxY1	10.03	11.90	10.47	13.42		
TrxY2	44.67	61.58	71.86	79.00	-0.69	
Nrx1	4.67	4.59	6.93	2.79		
Nrx2	100.30	65.51	75.52	43.24	0.41	
Nrx3	1.05	2.12	4.05	3.45	-1.95	
Nrx4	40.26	109.07	38.10	70.44		
VTC21	53.35	148.96	50.62	224.11		-0.59
VTC22	382.78	326.25	294.66	275.38		
VTC11	28.94	51.80	33.21	53.37		
VTC12	24.65	13.82	14.48	13.03	0.77	
GME1	0.63	2.34	0.56	1.50		
GME2	189.92	190.51	161.08	259.42		-0.45
GLDH	7.10	6.75	5.82	5.80		
VTC41	4.35	2.69	4.05	2.59		
VTC42	0.73	2.19	1.80	2.22		
GDH1/2	12.88	14.62	21.33	25.69	-0.73	
GDH3	3.33	4.16	4.71	5.28		
GULO1	9.24	5.38	8.10	5.95		
Miox1	27.12	33.21	9.64	16.80	1.49	
Miox2	3.19	3.96	4.29	2.01		
Miox3	16.75	40.82	9.48	23.67		0.79
GCL1	15.31	12.17	16.49	8.93		0.45
GCL2	19.79	76.48	16.64	32.15		
GS1	3.51	7.84	9.42	11.87	-1.42	-0.60
GS2	2.04	4.96	2.86	3.14		
VTE31	96.18	74.46	70.44	50.99		
VTE32	70.19	35.01	65.70	32.39		
VTE33	5.78	6.64	7.69	8.62		
VTE2	111.26	50.87	85.86	36.52	0.37	0.48
VTE1	27.22	34.56	33.84	29.77		
VTE4	0.66	3.50	0.43	7.13		
	0.00	50.00	300.00	[illegible]		

Figure 19. Redox-related genes expression in SP217 latex.

Discussion

ROS production and scavenging systems play an essential role in maintaining redox homeostasis in laticifers. *HbRBOH2* was the main ROS production genes expressed in latex of PB260, as well as in latex of SP217 (Figure 15). It was very low expressed in latex of SP217 compared with PB260. *CuZnSOD4* was main SOD gene expressed in latex of SP217 but not the main one in PB260, and it was expressed at higher level in SP217 than in PB260. NTR-TRX-Prx might be the main H_2O_2 scavenging pathway and GSH-GST might be the main oxidized products detoxification pathway in latex only based on the quantity and the abundance of latex expressing gene members involved and the abundance of their transcripts in latex. 13 *HbTRX* mRNA was very abundant in latex among 6 tissues studied and *HbTRXH5* was the most abundant expressing redox related transcript in PB260. Besides *HbRBOH2* and *CuZnSOD4* was observed, those genes involved in NTR-TRX-Prx, GSH-GST and antioxidants biosynthesis pathways had not so large fold change between two clones or were not abundant latex expressing genes (Figure 15). Clonal or tapping frequency differences might cause the large fold changes (0.44/6.56, 223.97/26.40 and 130.09/5.27) of *HbRBOH2* and *CuZnSOD4* observed between the two clones. A new experiment is needed to validate which factor lead to genes expression change.

Conclusion

Apart from plant model species, this study is the most complete genome-wide analysis of ROS production and scavenging systems and antioxidants biosynthesis in a perennial crop. *Hevea* has 407 redox-related genes, which is a higher number of genes compared to *Arabidopsis* and rice. In *Hevea*, 164 redox-related genes were expressed in latex of SP217 and 161 genes in latex of PB260, 13 genes were shown to be targeted by 11 microRNAs and 15 genes by 16 phased siRNA.

This study also revealed specific regulations of ROS-scavenging systems, which might be adapted to the strong and steady ROS production in latex cells due to recurrent harvesting stress and latex regeneration. HbRBOH2 was identified as the main source of ROS in latex. SOD activity is the first line of defence against anion superoxide, which play a crucial role to scavenge the ROS. *HbCuZnSOD4* might be the most important ROS scavenging genes to detoxify the ROS in latex of TPD tolerant tree. A new working model was proposed for transcriptional and post-transcriptional regulations in respect with predicted subcellular localization of deduced proteins. Conversely to *Arabidopsis*, none of the *Hevea* cytosolic SOD isoforms is subjected to posttranscriptional regulation by miR398. This data might support the maintenance of SOD activity and a consequent high level of anion superoxide dismutation.

General discussion and conclusion

1. A comprehensive analysis of redox-related genes in latex

Transcriptomics, degradome and sequence analyses led to predict their subcellular activities and posttranscriptional regulations through microRNAs. They highlighted critical steps of the redox homeostasis in latex cells and led to determine a list of priority studies for functional analyses. The objects of this PhD project was achieved (Figure 16). Comprehensive redox system in latex, gene modification and some molecular genetic marks will be our prospects in future research (Figure 16).

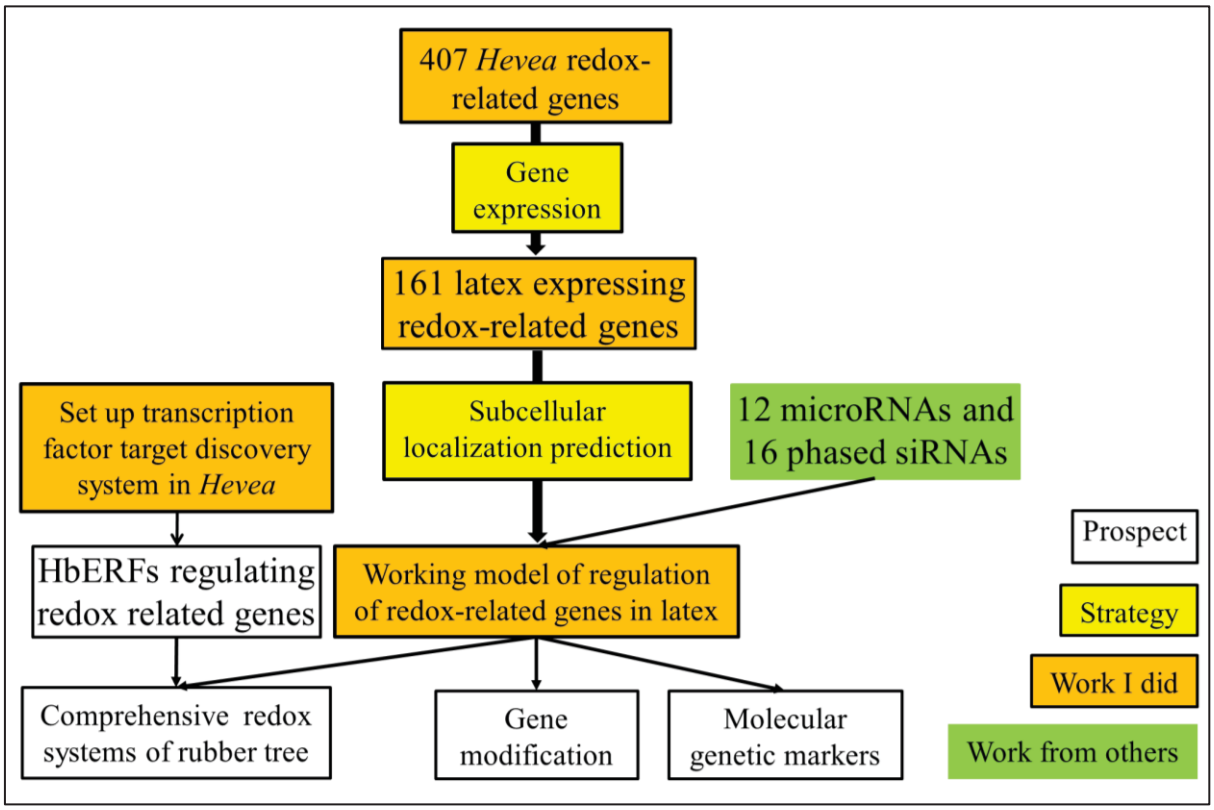

Figure 20. Scheme of general results and prosects for this project**.**

2. A new functional model for ROS and its' relevance to TPD in laticifer

Stress recognition induces the production of ROS by NADPH oxidases in both plants and animals (Kadota et al. 2015; Adachi and Yoshioka 2015). The lutoid membrane located NADPH oxidase generates species of toxic oxygen, which could be greatly activated by physiological concentrations of metallic cations and quinone-like compounds (Chrestin et al. 1984). Most of metallic cations such as Cu^{2+}, Ca^{2+} and Mg^{2+}, plastoquinone and ubiquinol were compartmentalized in lutoids normally (Jacob et al. 1993). They might act as activators or electron carriers for NADPH oxidase. Latex flows out from the laticifers with environmental and wounding stresses, as well as the stress from metabolic activity necessary for latex regeneration between two tappings. During the tapping activity on a healthy tree, there were a

shearing force on lutoids' membrane caused by latex flowing and an osmotic pressure decreasing caused by water replenishing from surrounding cells of laticifer. These two inevitable physical factors would have a consequent of lutoid lysis. Some released metallic cations would activate the NADPH oxidase and increase the production of ROS in laticifer. The oxidation of latex lipid, RNA, DNA and protein caused by ROS would happen if the ROS was not correctly and timely scavenged by CuZnSOD, catalase, peroxidases and peroxiredoxin in cytoplasm (Figure 17). In another hand, the cytosol phenol could be oxidized into quinone by hydrogen peroxide which would activate NADPH oxidase again (Figure 17). If the ROS scavenging enzymes could not manage these increased ROS in stressed tree, oxidation of lutoids membrane would extend and worsen. There would be an expansion of lutoids bursting which was accompanied by a ROS bursting in laticifer. Abundant coagulating factors released into cytoplasm results in latex coagulation in the bark of the stressed trees. This laticifer physiological dysfunctions syndrome was called dry-cuts syndrome. If the ROS bursting extend continually after latex coagulation, the inner bark spatially separated linamarin would be released. Released linamarase would convert it into cyanide which results in necrosis in the bark of stressed rubber tree (Figure 17).

To avoid dry-cuts syndrome and necrosis, ROS production has to be tightly controlled by ROS scavenging enzymes. CuZnSOD is the first line of ROS detoxification. Peroxidases and peroxiredoxin constitute the second one. Because of the low affinity for hydrogen peroxide of catalase (Clément et al. 2001), it is the third line which probably functions after the ROS bursting to prevent the necrosis while there is a higher concentration of hydrogen peroxide in laticifer. The reductive energy of peroxidases and peroxiredoxin comes from the NADPH. From some intermedia reactions, NADPH is consumed to reduce hydrogen peroxide. NRT and TRX form most important route to transmit the energy from NADPH to Prx or GPX (Figure 17). Laticifer generates ROS by starting from the oxidation of NADPH with oxygen. The energy for scavenging ROS and detoxify the electrophiles (ROS oxidized lipid, DNA and phenol) are also generated from the oxidation of NADPH. The overall reaction of the ROS production and ROS scavenging in laticifer is the oxidation of NADPH with oxygen. *Via* consumption of NADPH, the stress was recognized and responded by such a complex ROS production and scavenging homeostasis system in laticifer.

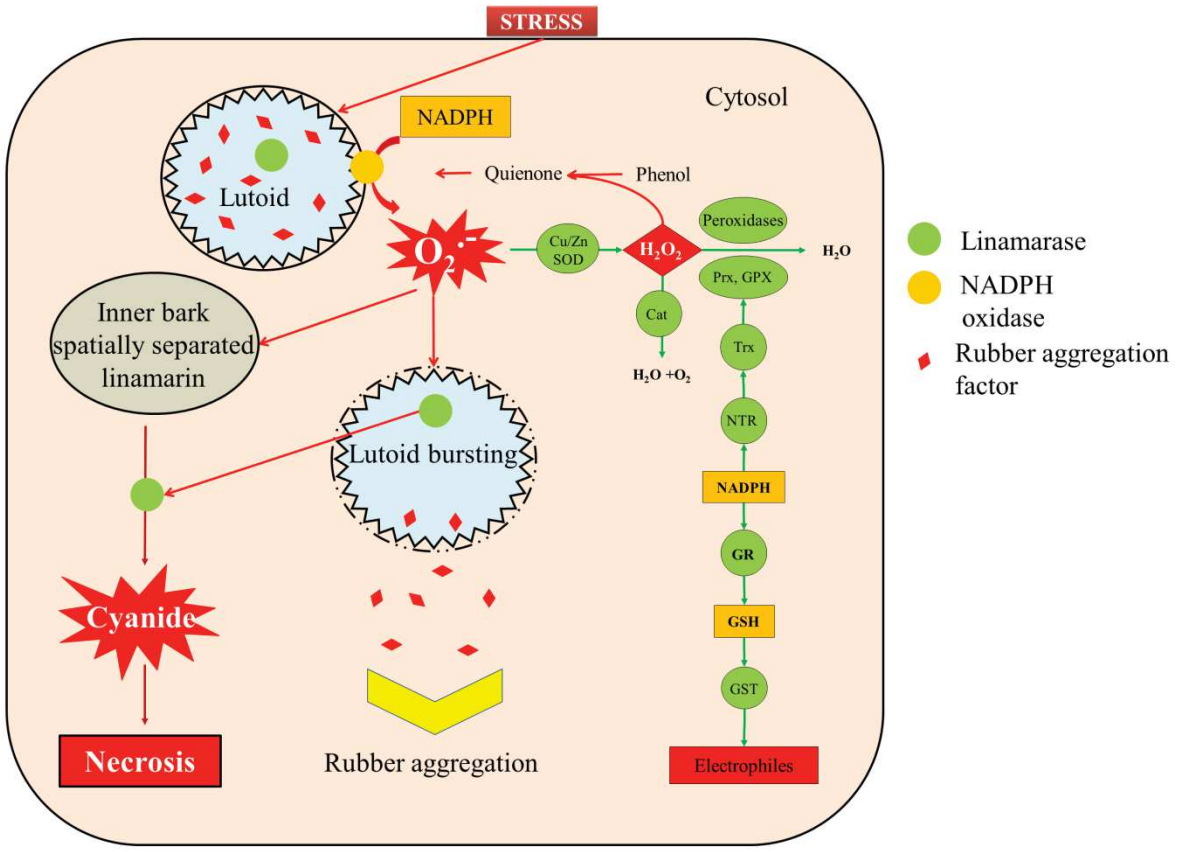

Figure 21. A model for ROS and its' relevance to TPD in laticifer.

3. Latex diagnosis for functional studies of TPD

Latex physiological parameters applied for research and agronomy are linked with latex flow and regeration between two tapping which influence the latex production. They are total solid content, lutoids brusting index (BI), total thiols content, magnesium content, sucrose content, pH, inorganic phosphorus content, redox potential (Eschbach et al. 1983; Jacob et al. 1989b). Latex BI and total thiols are related directly with ROS production and scavenging systems. BI, the percentage of lutoids lysis, can be tested *via* the ratio of the free acid phosphate in the cytosol (C serum of latex) to the latex total acid phosphate. Because of the acid phosphates are located in lutoids (Jacob et al. 1993). Lutoids brusting influence the latex flow. There is a highly significant inverse relation between BI and latex production (Jean d'Auzac 1989). Latex total thiols are mainly glutathione, cysteine and methionine which provide a reductive pool in latex (McMullen 1960). It is indispensable for ROS scavenging, ascorbate regeneration and electrophiles detoxification in latex. It maintains the stability of latex. Besides that keeps latex flow, thiols can function as activators for the key enzymes of latex metabolism, which maintains latex regeneration. For example, latex invertase can be activated by total thiols (Jacob et al. 1982). There is highly significant positive correlation between total thiols content and latex production (Jean d'Auzac 1989). All of latex physiological parameters apllied aiming to have a good yield of latex but no one to prevent TPD. Even the BI and total thiols may guide the exploiter to worry way in some cases. For example, the total thiols content increased about 5 folds while the latex production decreased 8% to 40% in low temperature season (Alam et al. 2003). Total thiols content increased about 2 folds and latex production increased markly in

long-term flowing tree induced by intensive tapping which takes a highrisk of TPD (Wei et al. 2015b).

Overaccumulation of ROS leads to TPD and affects latex production. Application of good diagnosis parameters to monitor redox homeostasis of rubber tree for TPD prevention will benefit the rubber tree exploiter. Total thiols concentration does not work for preventing TPD. Because total thiols includes reduced and oxidized thiols which cannot present the state of latex redox homeostasis. Although latex total thiols provide a reductive pool (McMullen 1960), reduced glutathione (GSH) acts as the main reductive molecule. GSH content or the ratio of GSH to glutathione disulfide (GSSG) can present the latex redox state effectively comparing to total thiols content. For that reason, GSH/GSSG was suggested to be the parameter of plant glutathione-linked responses in oxidative stress signaling rather than total thiols (Noctor et al. 2012). A drought sensitive barley strongly increased GSH and GSSG contents during water deficit while the ratio of GSH/GSSG was decreased (Marok et al. 2013). The ratio of GSH/GSSG will open avenues of research to improve the unreliable antioxidant parameter of the latex diagnosis.

4. Towards a gene network controlling redox homeostasis

4.1 Study interactions between transcription factors and redox-related genes

Transcription factors are important regulators of the cellular stress response and inducers of the ROS scavenging system in plant (Klotz et al. 2015). Low concentrations ROS control gene expression by acting as secondary messengers for cellular signalling (Turpaev 2002). The transcription factors are regulated by ROS level (Gupta et al. 1999; Law et al. 2013). Figuring out the interreactions between redox-sensitive transcription factors and redox-related genes will give a comprehensive knowledge of *Hevea* latex redox systems.

Chromatin immunoprecipitation sequencing (CHIP-seq), protein centered yeast one hybrid and TARGET (Bargmann et al. 2013) are the main genome-wide profiling interaction between TF and DNA. CHIP-seq (Park 2009) and protein centered yeast one hybrid (Moradpour et al. 2016) are only to figure out the transcription factor binding sites on genome which is not so informative after *Hevea* genome is available (Tang et al. 2016) and the binding site of TF is known. Although there are some false-positives and false-negatives from TARGET as well as the two others, the target genes can be further validated *via* cis-elements analysis of their promoters. With the available genome sequence, TARGET will be suitable for studying the target genes of TF, of which DNA binding sites are known.

4.2 Crosstalk between ROS and ethylene signalling pathway

Maintaining redox homeostasis plays a central role to regulate plant cell metabolism and development for adapting different environments. Plant hormones such as salicylic acid (SA), jasmonic acid (JA), abscisic acid (ABA), auxin and ethylene signaling pathways are involved in regulation of redox homeostasis (Chen et al. 1993; Mackerness et al. 1999; Kwak et al. 2006). Cross-talk between ROS and ethylene signling pathways regulate or control some plant environmental stress reactions. ROS played an important role in UV-B-induced ethylene synthesis in maize seedlings (Wang et al. 2006). H_2O_2-mediated stomatal closure could be

disrupted in the loss-of-function of ethylene receptor (*ETR1*) mutant (Desikan et al. 2005). T-DNA insertion in redox regulation transcription factor 1 (*RRTF1*) line showed greater sensitivity to photosynthetic stress compared to the wild type (Khandelwal et al. 2008).

Ethylene is the only one plant hormone reprted that can induce latex production and TPD, which indicated ethylene signalling pathway may be involved in regulation of redox system in *Hevea* latex to trigger TPD. *RRTF1* is main node of the redox responsive co-expression network that controls a regulon responsive to a change in redox status (Khandelwal et al. 2008). *HbERF-Xb1* was the only one orthologous of *RRTF1* in *Hevea*, was highly accumulated in latex after tapping and ethephon stimulation. The other ERFs candidates which involved in regulation of redox systems were illustrated (Zhang et al. 2017) in Chapter 1. Some ERFs might play a role in the redox regulatory network in *Hevea*, which have been the most intensively studied given the role of ethylene on rubber production: *HbERF-Ib5*, *HbERF-IXc4*, *HbERF-IXc5*, *HbERF-Xb1*, *HbERF-VIIa12* and *HbERF-VIIa17*.

4.3 Genome-wide transcription factor target discovery system for HbERFs based on stable transformation

For studying the target genes of ERF, TARGET is the most suitable technology for interaction study between *Hevea* ERF and target genes. The original TARGET experiment is a transient transformation system for genome-wide transcription factor target discovery (Bargmann et al. 2013), in which transformation of plant protoplast was essential. Transgenic technology of rubber tree callus is an efficient and robust technique in our group and the transgenic lines can give us more opportunity to observe the gene function deeply. This genome-wide transcription factor target discovery system will be adapted to *Hevea* transgenic callus lines of HbERFs, which can be routinely obtained by our team (Leclercq et al. 2010).

An adaption study of TARGET to rubber tree and apply this approach to study the targets of HbERFs is introduced (Figure 18). It was carried out and detailed in annex. Transcriptome analysis after promoter induction will allow the identification of primary and secondary target genes in the presence or absence of cycloheximide. GR allows the controlled entry of the chimeric GR-TF into the nucleus by addition of the GR-ligand dexamethasone (DEX). Beta-glucuronidase (GUS) gene was chosen as a control gene to support the reliability of the express system.

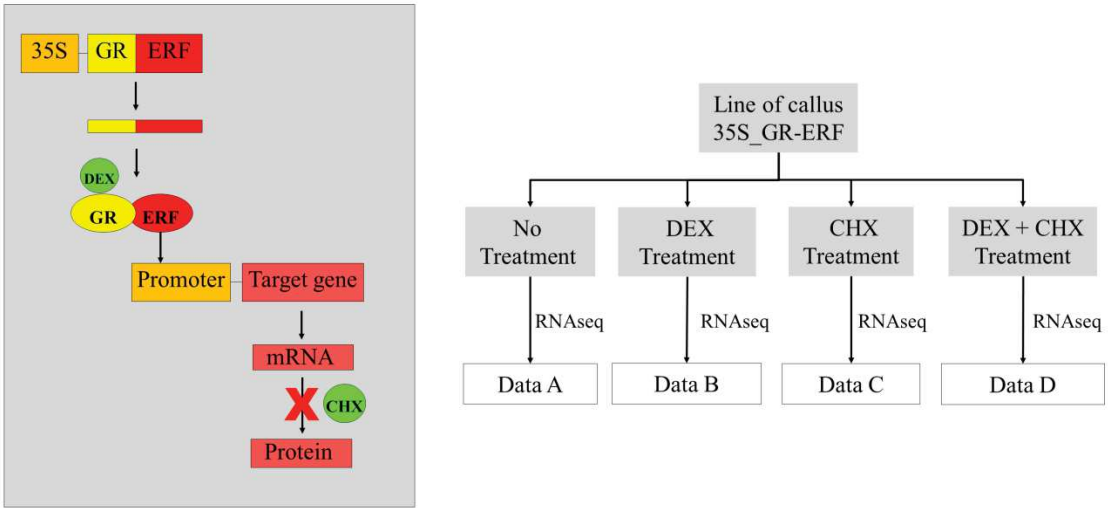

Figure 22. Identification of target genes of ERFs by TARGET system. DEX is an entry control to nucleus and activiation of target genes. CHX inhibits the protein tranlation. Data A. All the genes expressed at the basal levels. Data B. Target genes expressed at primary levels or secondary levels. Data C. All the genes expressed at basal levels. Data D. Target genes expressed at the primary levels.

4 Application of functional analyses in molecular breeding programmes

Research on functional analyses of redox related genes led to identify candidate genes closely related to important traits such as TPD tolerance. Breeding process of *Hevea* is time consuming and difficult because of non-synchronous flowering, heterozygous and long growth cycle of this tree. Developing the molecular genetic markers link to physiology characters is essential to shorten the breeding process. For this purpose, identification of random amplified polymorphic DNA markers for the dwarf character to avoid the wind damage was explored (Venkatachalam et al. 2004). 5883 putative single nucleotide polymorphisms (SNPs) were discovered *in silico* (Pootakham et al. 2011). 90 simple sequence repeats (SSR) markers were identified exhibiting polymorphism among the 18 rubber tree accessions based on a genomic shotgun sequences (Pootakham et al. 2012).

With the development of next generation sequencing, *Hevea* genome sequence was regarded to be the source to mining the molecular genetic markers. Since the first *Hevea* draft genome (1.1Gb) was published (Rahman et al. 2013) and the second *Hevea* genome (1.4 Gb) was reported (Yasukazu Nakamura 2014), two *Hevea* genome versions RRIM600 (1.55 Gb) (Lau et al. 2016) and Reyan 7-33-97 (1.37 Gb, N50 = 1.28 Mb) (Tang et al. 2016) were released later. Identification of functional gene families in genome wide was efficient (Li et al. 2014; Zou et al. 2015; Liu et al. 2016). These functional genes are potential molecular genetic markers. Another *Hevea* genome version was published recently (1.26 Gb, N50 = 96.8 Kb) (Pootakham et al. 2017). Those genome data give us good opportunity to develop the molecular genetic markers at the genome level. 14155 high quality SNPs were identified for supporting genetic improvement of latex yield and rubber trees growth (Chanroj et al. 2017).

Current challenges for rubber research and development with regard to redox systems will involve improving antioxidant capacity using natural genetic variability. Take those 5 genomes as references, especially the high quality one (Tang et al. 2016), make a SNP calling

at the genome level (Nielsen et al. 2011) and link redox related genes to densification genetic map locally will be done. TPD tolerant SNPs will be developed *via* SNP calling on 2 resequencing genomes and some transcriptomes of SP217 and PB260 (not published yet). Molecular genetic markers associated with high tolerance to oxidative damage in latex cells applied in breeding programmes will be hopeful to improve the rubber tree tolerance on TPD.

Annex

Genome-wide transcription factor target discovery system for *Hevea* ERFs based on stable transformation

1. Introduction

Identification of target genes of some ethylene response factors (ERFs) involved in the control of redox systems by a genome-wide transcription factor target discovery strategy is an example to study the regulators of latex redox system. *HbERF-Xb1* is the prior candiate to study. This annex was an adaption study of TARGET in rubber tree and applied this approach to study the targets of HbERFs. Beta-glucuronidase (GUS) gene was chose as control gene to support the reliability of the express system. HbERF-Xb1 was chose as an example to establish this system.

2. Materials and methods

2.1 Plant materials

The embryogenic friable wild type line of *Hevea* callus was established from integument-calli of clone PB260 and stocked in the lab by cryopervation in liquid nitrogen (Lardet et al. 2009). It was sub-cultured every 2 weeks on a maintenance culture medium (MM) for 6 cycles. The calluses was pre-cultivated for two weeks on a $CaCl_2$-free MM medium (Montoro et al. 2003) in glass tubes before Agrobacterium inoculation. After transformation, the transgenic calluses were sub-cultured on a decontamination medium (DM) every 3 weeks. The paromomycin contents in DM started with 0 mg/L in DM1 and DM2 to 50 mg/L in DM3, 75 mg/L in DM4, 100 mg/L in DM5, and were increased until to 150 mg/L. Callus treatments were made in a liquid medium (LD). The composition contents of these 3 mediums were listed in Table 1. The pH of all media was adjusted to 5.8 with KOH solution before autoclaving. Every plate for culturing contained 25 mL medium. Calluses were grown on the plates at an intensity of 30 aggregates per plate in a 27 °C dark room.

Table 7. The composition contents of MM, DM & LM.

Medium	MM	DM	LM
Composition	Final content		
NH_4NO_3	20 mM	20 mM	20 mM
KNO_3	20 mM	20 mM	20 mM
$MgSO_4$	3 mM	3 mM	3 mM
NaH_2PO_4	2 mM	2 mM	2 mM
$CaCl_2$	9 mM	9 mM	9 mM
H_3BO_3	150.08 µM	150.08 µM	150.08 µM
$MnSO_4$	100 µM	100 µM	100 µM

$ZnSO_4$	40 µM	40 µM	40 µM
$CuSO_4$	1.48 µM	1.48 µM	1.48 µM
Na_2MoO_4	0.99 µM	0.99 µM	0.99 µM
KI	5 µM	5 µM	5 µM
$CoCl_2$	1.01 µM	1.01 µM	1.01 µM
Inositol	300 µM		
Nicotinic acid	20 µM		
Vitamin A			
Pyridoxine-HCl	3 µM	3 µM	3 µM
Thiamine-HCl	2 µM		
Biotine	0.2 µM		
D-calcium pantothenate	1 µM	1 µM	1 µM
Ascorbic acid	1 µM	0.5 µM	0.5 µM
Choline chloride	0.1 µM		
L-cysteine-HCl	60 µM		
Glycine	5 µM		
Riboflavin	1 µM	1 µM	1 µM
Benzylaminopurine	1.35 µM	0.3 mg/L	0.3 mg/L
3,4-D	1.35 µM	0.3 mg/L	0.3 mg/L
$FeSO_4$	100 µM	100 µM	100 µM
Na_2EDTA	100 µM	100 µM	100 µM
$AgNO_3$	30 µM	-	-
Sucrose	234 mM	234 mM	234 mM
Abscisic acid (ABA)	0.5µM	0.5µM	0.5µM
Phytagel	2.3 g/L	3 g/L	-
Ticarcillin	-	500mg/L	500mg/L

Paromomycin	-	0 to 150 mg/L	-

2.2 Binary vectors and Agrobacterium strains

The fast restriction endonuclease and T4 DNA ligase were provided by New England BioLabs. DNA extraction kit was provided by Promega. Taq DNA Polymerase High Fidelity, Gateway LR reaction kits, *E.coli* DH5α and TOP10 competent cells were provided by ThermoFisher Scientific. Agrobacterium *EHA105* was stocked in our lab. The vector pCambia2300_GFP was constructed and stocked by our goup (Leclercq et al. 2010). The vector pBeaconRFP_GR was a gift from Gabriel Krouk (Bargmann et al. 2013). The full ORFs of *HbERF-Ib5*, *HbERF-IXc4*, *HbERF-IXc5*, *HbERF-Xb1*, *HbERF-VIIa12*, *HbERF-VIIa17* and *uidA* were cloned in vector pDONR^{TM}207 and their vectors were stocked by our group.

Vector pCambia2300_GFP was digested by *Sac I* and vector pBeaconRFP_GR was digested by *SacI*, *NaeI* and *NarI*. The digestion was carried out in 37 °C for 2 hours in a 50 μL reaction solution which contained 10 μg plasmid, 1μL fast restriction endonuclease, 5μL CutSmart buffer and water. After electrophoresis of the digested product in agarose gel, Vector DNA was extracted from the gel. Ligation was carried out 16°C overnight in a reaction solution which contained 1μL T4 DNA ligase, 1μL buffer, water, 50 ng pCambia2300_GFP backbone and 50 ng pBeaconRFP_GR backbone. 2μL ligation product was added into a 1.5 mL sterile tube to make transformation with 50 μL DH5α competent cells. *E.coli* transformation process was followed the protocol of competent cells kit. Spread transformation product on kanamycin selective plate and incubate the plate in 37°C overnight. Colony PCR was amplified with primer F1 (5'-TCTGCCGACAGTGGTCCC-3') and primer R1 (5'-GATGACCAAATGACCCTGCTA-3') by Taq DNA Polymerase High Fidelity. The amplification condition was 25 seconds denaturation at 94 °C, 25 seconds annealing at 59 °C, and 230 seconds elongation at 68 °C for 33 cycles. The plasmids were isolated from positive clones which were validated after visualization of PCR products separated on an agarose gel electrophoresis. These plasmids were further validated by *SmaI* digestion. Positive plasmid was sent to GATC sequencing company. The new vector constructed from pCambia2300_GFP and pBeaconRFP_GR was named pCambia2300_GFP_GR.

The candidate genes *HbERF-Ib5*, *HbERF-IXc4*, *HbERF-IXc5*, *HbERF-Xb1*, *HbERF-VIIa12*, *HbERF-VIIa17* and *uidA* were cloned into pCambia2300_GFP_GR vector from pDONR207 by LR reaction by following the Gateway LR reaction kit protocol. Colony PCR was amplified with primer F1 and primer R1. The binary vectors (pCambia2300_GFP_GR_ERFs and pCambia2300_GFP_GR_uidA) were introduced into *EHA105 Agrobacterium tumefaciens* strain by electroporation.

2.3 *Hevea* transformation and transgenic callus selection

Agrobacteria transformed by binary vectors were grown in liquid Lysogeny Broth medium which contained 50 mg/L kanamycin and 100uM acetosyringone at 28°C until OD_{600nm}=0.6. The bacteria were harvested by centrifugation and resuspended to OD_{600nm}=0.06 in liquid MM without Fe-EDTA, $CaCl_2$ or any growth regulators but with 100μM

acetosyringone (Leclercq et al. 2010). The wild type calluses were immersed adequately into OD_{600nm}=0.06 bacteria mixture and took out immediately. Calluses and agrobacteria inoculations were 3 and 4 days respectively in 20°C of dark condition. Every transformation experiment was started with 10 tubes (5 tubes for 3 days and 5 tubes for 4 days). 60 aggregates were picked out from every tube and grown on 2 DM1 plates after inoculations.

GFP fluorescence activities of the fresh calluses were observed under microscope (MZ FLIII, Leica Microsystems, Wetzlar, Germany) every 3 weeks before sub-culturing. GFP positive calluses were selected before sub-culturing. In the first selection, 50 GFP positive aggregates (25 aggregates from 3 days inoculations and 25 aggregates from 4 days inoculations) were selected to establish independent lines for every transformation event.

2.4 Glucuronidase assay (GUS staining)

The calluses (GR_ERF-Xb1 and wild type) were bathed in a solution containing 50 mM sodium phosphate buffer (pH=7.0), 2 mM EDTA, 0.12 % Triton, 0.4 mM ferrocyanide, 0.4 mM ferricyanide, 1.0 mM 5-bromo-4-chloro-3-indoxyl-beta-D-glucuronide cyclohexylammonium salt (X-Gluc). They were treated under vacuum 10 minutes for 3 times. The calluses were incubated at 37 °C and let overnight for coloration. The calluses will be viewed and photographed directly with camera.

2.5 Cycloheximide (CHX) and dexamethasone (DEX) treatment

To test if CHX works in *Hevea*, calluses (GR_ERF-Xb1) were treated in 40 µM CHX contained LM at 4 different lengths of time (0, 0.5, 1 and 4 hours). After CHX test, the calluses (GR_ERF-Xb1) were treated with CHX and/or DEX at concentration of 40 µM and 10 µM respectively. 50mM CHX stock was dissolved in dimethylsulfoxide and 1mM DEX stock was dissolved in ethanol. The calluses (GR_ERF-Xb1) were collected from the 4 plates into 50 mL sterile tubes together with 20 mL liquid nutrient medium (ENT). The callus mixture was separated into 4 sterile tubes (50 mL) equivalently. The calluses in 4 tubes were treated by CHX + DEX, CHX, DEX and blank control respectively. CHX + DEX and CHX tubes were added 4µL CHX solution, meanwhile DEX and blank control tubes were added 4µL dimethylsulfoxide. The 4 tubes were incubated in a shaker at 70 rpm and 28°C for 1 hour. And then, CHX + DEX and DEX tubes were added 50 µL DEX solution, meanwhile CHX and blank control tubes were added 50 µL ethanol. The 4 tubes were incubated in a shaker at 70 rpm and 28°C for 16 hours. The calluses were freezed in liquid nitrogen after removing the solutions in the tubes. They were stocked in -80°C for protein or RNA isolation. All of these steps were carried out in sterile bench.

2.6 Western blot

The protein of those treated calluses was isolated to check the degradation western blot. Protein extraction buffer was prepared (Table 2) and kept in 4°C. 200 mg grinded powder of callus was added into 510 µL cold extraction buffer and mixed adequately. Centrifugation was made by 15 min at 4°C at 15000 rpm. The supernatant was centrifuged again in a new tube at

same parameters. Final supernatant was proceeded to protein content determination or/ and kept at -20°C

Table 8. Protein extraction buffer preparation for 102 mL.

Composition	Quantity
0.5 M Na_2EDTA, pH=8	2 mL
N-Lauroylsarcosine	100 mg
Triton X-100	100 µL
50 mM Phosphate Buffer, pH=7	100 mL

Protein content determination was quantified *via* a BSA (Serum albumin bovine) standard curve. BSA was dissolved in 9% sodium chloride with sodium azide. Concentrations of standard solutions were 125, 250, 500, 750, 1000, 1500 and 20000 µg/mL. Quantification was made in a 96-well plate which was flat bottom (Greiner bio-one). The quantification of BSA standard from Pierce (Pierce Bovine Serum Albumin Standard Pre-Diluted Set) was duplicated. Water was a blank control and was quantified with samples at the same time. 2 µL samples and 198 µL Bradford solution were added into one well of the plate. Samples were mixed with a plate shaker for 30 seconds and equilibrated at room temperature. The plate was removed from shaker and incubated for 10 minutes at room temperature before detection. OD measurement was made with Nanoquant at 595 nm. With the BSA standard, the linear regression of the standard curve was calculated. With the equation, protein contents of all the samples were determined. If a sample was too concentrated, a dilution and another quantification of the sample would be made.

40 µg of denatured protein was run on a pre-cast acrylamide gel following the manufacturer instructions (ladder, and denaturing buffer). The electrophoresis was arranged around 25 minutes at a voltage of 200 volts. Proteins were transferred on the membrane with the iBlot system *via* P0 program (nitrocellulose transfer stack) for 7 to 8 minutes. Proteins on the membrane were checked with Ponceau coloration before western blot. To prepare Ponceau coloration solution, the Ponceau powder was dissolved in 1% acetic acid (HAC) solution at concentration of 0.05 g/L.

All antibodies were provided by Sigma. Actin is stable protein in plant cell. NPTII and GR domain are short half-life proteins in plant cell. Antibodies' application content for western blot was 1/500 to 1/2000. The primary antibody and second antibody binding reactions and the revelation were followed the manufacturer instructions.

2.7 Total RNA isolation

Total RNA were isolated from the latex by an adapted method (Kush et al. 1990a). Cesium chloride (CsCl) was dissolved in NaAc solution (3M & pH=6) at concentration of 5.7 M. The RNA extraction buffer was prepared following the Table 3. 9.5mL extraction buffer was dispensed into every 50 mL sterile centrifuge tube. 1 to 2g grinded samples by the chilled

mortar was added into the same tube. 30 seconds of vortex was to mix well. And the vortex was repeated another 2 times every 3 minutes. Centrifugation parameters were 30 minutes at 4°c and 10000g. Ultracentrifuge tubes were prepared with distributing 3mL CsCl solution. These suspend phases was add into in those tubes with CsCl. Centrifugation parameter were 20 hours at 20°C and 32000 rpm. The RNA pellets were washed with 70% ethanol and dissolved in sterile H_2O. RNA samples were stored in -80°C freezer.

Table 9. RNA extraction buffer preparation.

Sterile 5M guanidine thiocyanate & 3M NaAc (pH=6)	48 mL
Sterile 20% sarcosine	2.64 mL
Sterile 10% PVP	5.4 mL
Sterile H_2O	2.52 mL
β mercaptoethanol	600 μL

2.8 RNA quality test and real time-PCR analysis

GR-F: GAACCCGAGGTGTTGTATGC
GR-R: GCCTGGTATCGCCTTTGC
Check the expressions of GR_ERF-Xb1
RNA Precipitation was carried out with 1/10 volume of 3M NaAc, 0.3 μg of glycogen per 1μL of aqueous sample and 3 volumes of cold 100% ethanol. And

3. Result

We rebuilt the pBeaconRFP_GR vector with pCambia2300_GFP vector to make it more suitable for transformation of *Hevea* callus. In the new vector, pCambia2300_GFP_GR, *HbERF-Xb1* was fused expressed with glucocorticoid receptor (GR) in *Hevea* callus.

After 6 or 7 cycles of GFP activity selection, some aggregates were fully GFP positive and were considered as established transgenic lines.

Table 10. Transgenic lines obtained

Transformation event	Genes in binary vector	Lines NO.
TS32	35S_GFP, GR_ERF-Xb1	6
TS34	35S_GFP, GR_GUS	3
TS35	35S_GFP, GR_ERF-IXc4	10
TS36	35S_GFP, GR_ERF-IXc5	8
TS38	35S_GFP, GR_ERF-Ib5	4

TS39	35S_GFP, GR_ERF-VIIa12	4
TS40	35S_GFP, GR_ERF-VIIa17	5
TS41	35S_GFP, GR_ERF-IIb2	10

The GUS staining results prove our transgenic expression system is working in *Hevea* callus (Figure 15).

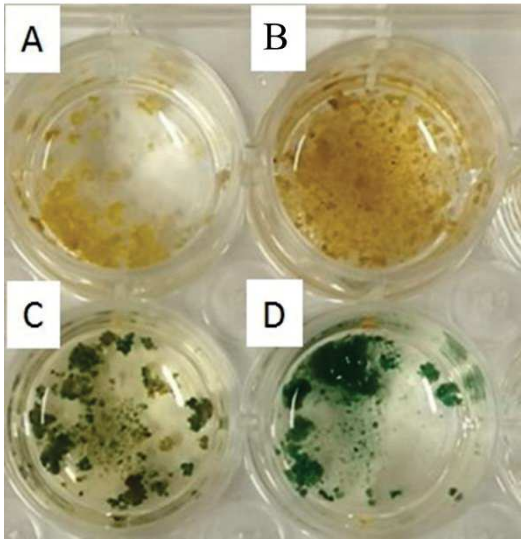

Figure 23. GUS staining. Well A and B are wild type *Hevea* calluses. Well C and D are two independent GR_GUS transgenic *Hevea* calluses.

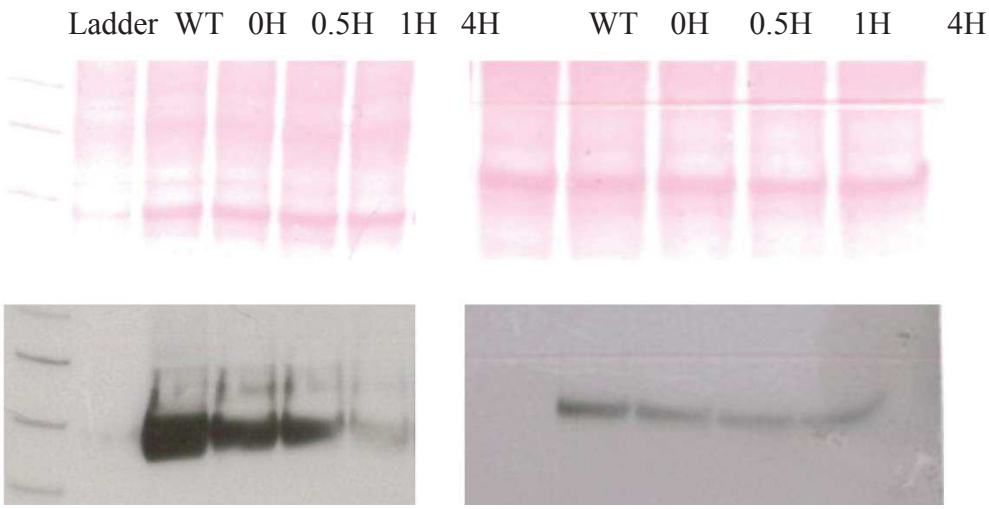

Figure 24. CHX testing.

Hevea transgenic calluses (GR_ERF-Xb1) were treated with CHX and DEX at concentration of 40 µM and 10 µM respectively. The calluses were collected from the 4 plates into 50 mL sterile tubes together with 20 mL liquid nutrient medium. This mixture was separated into 4 sterile tubes (50 mL) equivalently. The calluses in 4 tubes were treated by CHX + DEX, CHX, DEX and blank control respectively. They were freezed in liquid nitrogen after removing the solutions in the tubes and were stocked in -80°C for RNA isolation.

We have sent the RNA samples to sequencing company twice. But this RNA samples were degraded based from their feed backs. We will send the RNA samples with the good quality of RNA samples (Figure 2) again by dry ice as soon as possible.

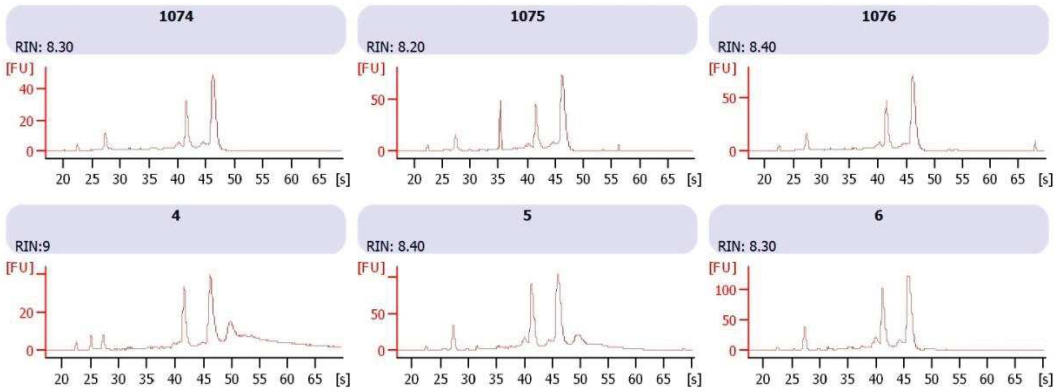

Figure 25. RNA quality testing.

Vector	Lines	Treatment	GR/RH2b
GR_ERF-Xb1	A06	CHX+DEX	0.70
GR_ERF-Xb1	A06	CHX	0.62
GR_ERF-Xb1	A06	DEX	0.12
GR_ERF-Xb1	A06	-	0.05
GR_ERF-Xb1	A11	CHX+DEX	0.92
GR_ERF-Xb1	A11	CHX	1.02
GR_ERF-Xb1	A11	DEX	0.11
GR_ERF-Xb1	A11	-	0.15
GR_ERF-Xb1	A23	CHX+DEX	0.29
GR_ERF-Xb1	A23	CHX	0.27
GR_ERF-Xb1	A23	DEX	0.01
GR_ERF-Xb1	A23	-	0.02
GR_ERF-Xb1	A25	CHX+DEX	0.17
GR_ERF-Xb1	A25	CHX	0.20
GR_ERF-Xb1	A25	DEX	0.07
GR_ERF-Xb1	A25	-	0.07

Figure 26. Real-time PCR to check the expression of insertional HbERF-Xb1 in transgenic calluses.

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